INNATE AND ADAPTIVE IMMUNE RESPONSES TO MEASLES AND THEIR ASSOCIATION WITH VIRAL CONTROL AND CLEARANCE

by
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Abstract

Measles remains one of the most important causes of morbidity and mortality among children worldwide, despite the availability of a safe and effective vaccine. Measles virus, the etiologic agent of measles, is a member of the *Paramyxoviridae* family and has a negative-sense single-stranded RNA genome. The host immune response to measles is essential for viral clearance, recovery, and the establishment of long-term immunity. Paradoxically, measles is also associated with a transient state of immune suppression. In order to better understand the complexities of this disease we utilized the highly relevant rhesus macaque model.

Use of the rhesus macaque model allowed us to study viral clearance, investigate the innate immune response, and characterize the dynamics of the adaptive immune response during a primary, wild-type measles virus infection. We show that viral RNA persists in multiple tissues and immune cells months after infectious virus is cleared. Additionally, we present a case that confirms that T cells play a vital role in viral clearance even in the absence of a neutralizing antibody response.

The innate response to measles is particularly difficult to study in humans because the clinical diagnosis relies on the presence of a rash, which is a hallmark for the onset of the adaptive immune response. Using our rhesus macaque model, we were able to show that innate immunity to measles does not involve type I IFN production, indicating that activation of the adaptive immune response relies on other innate immune factors. We were further able to characterize the dynamics of the adaptive immune response to measles. Our work shows that the humoral

immune response to measles virus involves the rapid production of virus-specific antibodies however, maturation of these responses are slow. We also demonstrate that virus-specific T cell responses are much more prolonged than previously recognized. This work provides new insight to our understanding of measles-induced immune suppression, the development of long-lived immunity, and occasional CNS complications due to viral persistence. Lastly, we analyzed the cellular immune response to a third dose of MMR and showed minimal boosting in immunity.

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Chapter 1.

General Introduction

Measles is a highly contagious, vaccine-preventable illness caused by the measles virus. Previously unexposed humans and non-human primates are susceptible to disease. Upon infection, virus travels to lymphoid tissue and then to multiple organs resulting in a systemic infection. Clinically, the onset of rash occurs 10-14 days after infection and coincides with the appearance of the adaptive immune response. Infection within the host is characterized by a robust virus-specific immune response leading to eventual recovery and establishment of life-long immunity. Paradoxically, measles virus infections are also associated with a transient period of immune suppression leading to increased susceptibility to secondary infections. Recent strides have been made toward global measles control through the use of mass vaccination campaigns, however various logistical and financial barriers have hindered sustained vaccine efforts. In addition there has been a recent resurgence of measles in many industrialized nations, mainly due to objections to vaccination based on complacency or various philosophical and religious beliefs. The World Health Organization (WHO) estimated that approximately 134,200 people, mostly children under the age of five, died of measles or associated sequelae in 2015.

1.1 Virology

Measles virus (MeV) is an enveloped, single-stranded, negative-sense RNA virus. MeV is a member of the morbillivirus genus and *Paramyxoviridae* family [1]. The measles genome is approximately 16 kb and encodes for six structural and two non-structural proteins [1] (Figure 1-1). The hemaglutinin (H) protein is a type II transmembrane glycoprotein that is an important determinant of morbillivirus tropism [2]. H binds to cellular receptors and interacts with the fusion (F) protein to mediate fusion of the viral envelope with the host cell membrane for viral entry. Fusion occurs via a conformational change in both proteins triggered by H binding to cell-surface receptors [3]. The matrix (M) protein interacts with the cytoplasmic tail of H and F glycoproteins, modulates the targeting and fusogenic capacity of envelope glycoproteins, and directs virus release from the apical surface of epithelial cells [4]. The nucleocapsid (N) protein is typically the most abundant protein and is required to encapsidate viral RNA for replication and transcription. The N-terminal domain of this protein is required for virus assembly and packaging [5, 6]. The phosphoprotein (P) and the large (L) protein function in association with each other for the virus' RNA-dependent RNA-polymerase [7]. The P gene encodes two additional MeV non-structural proteins C and V, which are important modulators of the host IFN response [8].

Currently there are three known cellular receptors for MeV: CD46 [9, 10], CD150 (or SLAM) [11], and nectin-4 [12]. CD46 is a human complement regulatory protein expressed on all nucleated cells and preferentially on the apical surface of polarized cells. There are four isoforms in humans, all of which MeV can utilize as receptors. In addition, monkeys have a CD46 homolog that can be utilized by MeV [13]. Signaling lymphocyte

activation molecule (SLAM or CD150) is a glycoprotein expressed on cells of the immune system: immature thymocytes, activated T and B-lymphocytes, activated monocytes, and mature dendritic cells. Most MeV vaccine strains can utilize CD46 efficiently, while wild-type (WT) strains do not. In contrast, both WT and vaccine strains can use SLAM as a receptor however the affinity and efficiency differ. The distributions of CD46 and SLAM in tissues don't account for MeV replication in epithelial cells (in vivo or vitro). Poliovirus receptor-like 4 (nectin 4) is an adherens junction protein of the immunoglobulin superfamily and has been identified as the receptor located on basolateral side of epithelial cells [12].

1.2 Epidemiology

Measles is a highly contagious respiratory disease that caused millions of deaths annually before the introduction of an effective vaccine in the1960's. Due to its high infectivity, sustained vaccine efforts are important for interruption of the transmission cycle. The basic reproductive number (R^0), or the mean number of secondary cases that arise if an infectious individual is introduced into a completely susceptible population, is estimated to be 12 to 18 for measles [14]. Based on this high R^0, the level of population immunity (herd immunity) necessary to interrupt measles transmission is between 93 to 95%.

The dynamics of measles outbreaks in populations have been very well characterized. When endemic, measles incidence typically displays a temporal pattern characterized by annual seasonal epidemics superimposed on longer 2-5 year epidemic cycles [15].

Seasonal fluctuations in measles transmission are generally correlated with fluctuations in population density [16].

Humans are the only reservoir for measles virus. There is also no evidence of latent or persistent infections that confer long-term contagiousness. Thus, measles eradication is considered feasible. While significant progress has been made in reducing global measles incidence, the requirement for high levels of herd immunity makes control efforts more difficult. In addition, there are a number of logistical and financial challenges that need to be met for eventual measles eradication to be achieved.

1.3 Pathogenesis and Pathology

Measles is transmitted from person to person via respiratory droplets or aerosols. After the introduction of virus into the respiratory tract, alveolar macrophages and dendritic cells become infected and transport virus to draining lymphoid tissues. Once transported to lymphoid tissue the immune response is initiated, virus is amplified, and then spreads systemically to infect multiple organs [17-19]. Infected immune cells (B cells, CD4+ and CD8+ T cells, monocytes) are primarily responsible for viral dissemination to various tissues including lymph node, spleen, liver, lungs, and skin [20, 21]. There is an incubation period of 10-14 days and a 2-3 day prodrome of fever, cough, and conjunctivitis followed by the appearance of the prototypical maculopapular rash (Figure 1-1). Individuals are most infectious from 5 days before through 5 days after the onset of rash [22]. In addition, the onset of rash coincides with the appearance of adaptive immune responses and initiation of virus clearance[22]. While infectious virus is

typically cleared by 2-3 weeks post-infection, the clearance of viral RNA is much slower [23].

In immunocompetent individuals, infection typically results in a robust virus specific immune response, which allows them to eventually recover. However, the transient period of immune suppression cause by measles can result in susceptibility to opportunistic infections, which result in most measles associated morbidities and mortalities. In rare cases, measles can establish persistent infection in the CNS leading to the development of measles inclusion body encephalitis (MIBE) or subacute sclerosing panencephalitis (SSPE), which may occur months to years after acute MeV infection [24]. Lastly, recovery from infection typically results in the acquisition of life-long immunity to reinfection.

1.4 Host Immune Responses to MeV

The immune responses to MeV are important for virus clearance, recovery from infection, and protection from re-infection and are also responsible for several of the clinical manifestations of disease. The roles of various components of the immune response in recovery have been mainly determined from documented outcomes of natural measles infection of patients with deficiencies in immunologic function. However, our more detailed understanding of the importance of various immune components have come from experimental infection of macaques, which allow for longitudinal studies of virus-specific immune responses and depletion of specific components of the immune system. In general, deficits in antibody production permit recovery, while deficits in cellular immune responses may lead to slowed clearance and progressive disease.

Innate Immune Response

The innate immune system provides an immediate first line of defense against infections. In short, this immune response is triggered in a cell intrinsic manner when pattern recognition receptors (PRRs) within the infected cell sense the virus and trigger downstream signaling cascades that activate immunity. The activation of innate immune responses can not only act directly to suppress viral replication and dissemination to other cells, but is also critical for programming subsequent adaptive immune responses. Many viruses have evolved ways to inactivate various innate immune signaling factors in order to escape immune surveillance, thus, determining the role and importance of specific components of the innate response during infection can be difficult.

In vitro studies have shown that MeV RNA or proteins interact with PRRs and can activate NFkB and IRF-3 signaling pathways in a strain-dependent and cell-type specific manner. Epithelial cells show activation of NFκB and AP-1 (activator protein-1) and increased production of the chemokine CXCL8 (IL-8) in response to MeV infection [25, 26]. However, the NFκB pathway and tumor necrosis factor-α (TNF-α) production are suppressed in acute and persistently infected monocytes, potentially as a result of the up-regulation of the ubiquitin-modifying enzyme TNFAIP3 (A20), a negative regulator of NFκB [26-28].

Some inflammatory cytokines and chemokines are induced *in vivo* during measles. Levels of TNFα, IL-6, IL-8, IL-1β and IL-18 are increased in plasma of children during measles with elevated levels of TNFα and IL-1β being associated with in-hospital mortality [29-32]. IL-1β mRNA and protein are increased in MeV-infected monocyte-derived cells and in PBMCs isolated from patients after rash onset [27, 28, 33].

Additional *in vitro* studies suggest that MeV infection leads to NLRP3 inflammasome activation, resulting in caspase-1 mediated maturation of IL-1β [34]. The production of inflammatory chemokines is important for cell homing to infected tissues. Levels of chemokines CCL2, CCL4, CCL13, CXCL8 and CXCL10, are elevated in children with measles, and levels were higher in children who died in-hospital compared to those who survived to discharge [32].

Type I IFN (IFNα/ IFNβ) and Type III IFN (IFNλ, IL28a, IL-28b, IL-29) can be induced as part of the innate immune response to viral infection. IFN induction typically occurs when signaling pathways are activated after virus recognition by PRRs such as toll-like receptors and RNA helicases. Both Type I and III IFNs activate the JAK-STAT signaling pathway and lead to the induction of IFN-stimulated genes, which encode for various proteins that can inhibit viral replication. *In vitro* and *in vivo* studies suggest that in the absence of defective interfering RNAs, neither WT nor vaccine strains of MeV efficiently induce Type I or III IFN [35]. The RNA helicases, RIG-I and MDA-5, are cytosolic PRRs that sense viral RNA in infected cells, interact with a common adaptor MAVS (also known as VISA/Cardif/IPS-1) and ultimately activate signaling pathways that lead to Type I IFN induction (Figure 1-3).

The MeV C and V proteins, encoded within the P gene, appear to be the main viral proteins that prevent the induction of IFN pathways [36]. The MeV V protein has been shown to block IFN induction by directly binding to MDA-5 (Figure 1-2) [36]. Subsequent studies demonstrated that the V protein is able to inhibit MDA-5 activation by a forming a complex with PP1α/γ thereby preventing MDA-5 dephosphorylation (Figure 1-2) [37, 38]. Additionally, MeV can also target PP1 in dendritic cells (DCs) by

interacting with DC-SIGN. MeV binding to DC-SIGN signals the activation of Raf-1, which leads to the inhibition of PP1 and suppresses RIG-I and MDA-5 activation (Figure 1-2) [39]. Alternatively, the V protein of MeV can prevent IRF-7 mediated activation of type I IFNs by binding IKKα (Figure 1-3) [36]. The MeV C protein has a more indirect effect on type I IFN induction. The C protein has been shown to be a negative regulator of viral RNA synthesis and thus acts to inhibit IFN induction via blocking the production of PAMPs [8].

Humoral Immune Response

Measles-specific humoral immune responses are important for protection and may contribute to recovery from infection. Antibodies to measles virus are sufficient for protection because infants are protected by passively acquired maternal antibodies, and administration of anti-measles immune globulin partially protects children from measles after exposure [40]. Humoral immunity seems to play a very limited role in viral clearance as transient depletion of CD20+ B cells in macaques does not affect clearance of infectious virus [41]. However, in MeV-infected individuals, antibody-dependent cellular cytotoxicity correlates temporally with clearance of cell-associated virus from the blood [42].

Antibody production is dependent on the activation and differentiation of B cells. B cells become activated upon the binding of antigen to the B cell receptor (BCR) via interaction with activated helper T cells. Upon activation, B cells proliferate and can differentiate into antibody secreting cells (short- or long-lived) or memory B cells. MeV-specific antibodies are first detectable at the onset of rash. Measles-specific IgM appears

within 72 hours after rash onset and is maintained for 28 days, while IgG responses are generated shortly after and are maintained for decades. The IgG isotype is initially IgG2 and IgG3 followed by a switch to predominantly IgG1 and IgG4 in the memory phase [43]. IgA, IgM, and IgG antibodies to MeV can not only be detected in serum but also in nasopharyngeal secretions and saliva, thus providing the opportunity to use noninvasive methods for determining immune status.

MeV-specific antibodies are produced to most MeV proteins but appear first and most abundantly to N, then H and F, respectively. MeV-specific neutralizing antibodies correlate with protection and play a key role in preventing subsequent MeV infections. The majority of neutralizing antibodies are specific for the H glycoprotein, however F-specific antibodies also display some neutralizing activity [44-46]. Neutralization is generally measured by plaque reduction of the Edmonston strain of MeV on CD46-expressing Vero cells.

Cellular Immune Response

Several lines of evidence suggest that cellular immunity may be important for measles virus control and clearance. Children with agammaglobulinemia recover from measles while those with impaired cellular immunity often develop progressive infection of the lungs and CNS [47, 48]. During the acute phase of disease, the appearance of cellular immune responses tends to coincide with the onset of the rash (Figure 1-4). Biopsy of the rash shows infiltration of CD4+ and CD8+ T cells in areas of MeV-infected epithelial cells, demonstrating that the rash is a manifestation of the cellular immune response [49]. Studies involving experimental infection of macaques has allowed for

longitudinal analysis of the MeV-specific cellular immune response however, the relative contributions of specific subsets of T cells to protection and immunity are not completely understood.

The two major subsets of T cells are CD4+ and CD8+ T cells. During measles, both CD4+ and CD8+ T cells are activated and expanded. CD8+ T cells, also known as cytotoxic T cells, function to eliminate virally infected cells. CD8+ T cells are considered to be most important for the control and clearance of MeV. Depletion of CD8+ T cells in macaques resulted in more severe symptoms and prolonged viremia compared to control macaques [49]. Additionally, ex vivo stimulation of PBMCs with MeV-specific peptides have shown that CD8+ T cells are expanded, have cytotoxic activity, and produce IFNγ demonstrating that effector memory is established during infection [50-54].

T-helper (CD4+) cells play a central role in immune protection and have various immune functions depending on the cytokines they produce. Upon encountering antigen in the context of MHC class II on the surface of antigen presenting cells, naïve CD4+ T cells become activated and can differentiate into different subsets: Th1, Th2, Th9, Th17, Th22, regulatory T cells (Tregs), and follicular helper T cells (Tfh). Th1 cells produce IFNγ, IL-2, and TNFα, and mediate immune responses against intracellular pathogens. Th2 cells produce IL-4, IL-5, IL-10, and IL-13 that are important for mediating host defenses to extracellular parasites and B cell proliferation. Treg cells play a critical role in maintaining self-tolerance as well as regulating immune responses via the production of IL-10 and TGFβ.

T-helper type 1 (Th1) responses tend to be predominant during the acute phase of measles predominantly producing IFNγ and IL-2, while the convalescent phase of

infection appears to be skewed towards T-helper type 2 (Th2) and regulatory T cell (Treg) responses associated with the production of IL-4, IL-10, and IL-13 [55-58] (Figure 1-4). Early production of IFNγ may be important for controlling virus spread as it has been shown to suppress MeV replication *in vitro* in epithelial and endothelial cells [59] and *in vivo* in the brains of infected rodents [60, 61]. The shift to the production of type 2 cytokines is likely to promote B cell proliferation and contribute to the continued production of antibody-secreting cells. Tregs are induced during measles, however their exact role in pathogenesis is still unknown. Detection of CD4+ CD25+ Tregs has been reported in humans and SLAM-expressing mice during measles virus infection [62, 63]. Furthermore, MeV-infected macaques have sustained upregulation of Foxp3, a Treg-specific transcription factor, after clearance of infectious virus [23]. A role for Th17 and Tfh cells in measles has not yet been described.

The humoral immune response is typically the primary focus when assessing vaccine efficacy and protection from measles, and most studies largely correlate serum antibodies with protection [64]. However, a few studies using both wild-type and vaccine strain measles viruses demonstrate that insufficient or impaired cellular immune responses may lead to delayed clearance of MeV RNA and progressive or even fatal infection [65]. These observations suggest that host immunity and MeV control and clearance are a complex orchestration between the cellular and humoral arms of the immune response.

1.5 MeV-induced Immune Suppression

The paradox of measles is that the acute phase of infection is not only associated with immune activation and a robust virus-specific adaptive immune response that leads

to recovery and lifelong immunity, but also with profound immune suppression. The immunosuppressive effects of measles were first recognized in 1908 by von Pirquet who described the loss of delayed type hypersensitivity responses to tuberculin in MeV-infected individuals [66, 67]. While the exact mechanism of MeV-induced immune suppression is not completely understood, several factors have been proposed to contribute to this phenomenon: (1) lymphopenia, (2) functional impairment of immune cells, (3) imbalance of cytokine production, and most recently (4) depletion of CD150+ memory lymphocytes [68, 69].

The acute phase of MeV infection is associated with decreased numbers of T cells and B cells in circulation and lymphoid tissue [70-72]. This loss of mature lymphocytes due to infection is likely to contribute to lymphopenia. The MeV receptor, CD150, is expressed at high levels by activated $CD45RO^{high}$ memory T-lymphocytes and a subset of B-lymphocytes that are preferentially depleted during infection [18, 73, 74]. Altered trafficking and increased susceptibility to cell death are also likely to contribute to a decrease in lymphocytes [70, 75, 76]. T cell numbers return to normal after 10 days once clinical symptoms have subsided [31, 70, 72]. Therefore, it is likely that lymphocyte depletion in the periphery plays only a minimal role in prolonged measles-induced immune suppression.

Various subsets of DCs are susceptible to MeV infection. Infection of DCs *in vitro* results in the loss of the ability of these cells to stimulate the proliferation of naïve CD4+ T cells in a mixed leukocyte reaction [77]. CD40-activated DCs show enhanced replication of MeV. Furthermore, enhancing replication of MeV during DC-T cell interaction leads to a decrease in the production of IL-12, decreased capacity for T cell

proliferation, and massive cell death [78]. Therefore, it is possible that MeV-induced immune suppression may result from impaired DC function and antigen presentation. However, *in vivo,* strong MeV-specific immune responses are developed resulting in rapid clearance of infectious virus and establishment of life-long immunity.

Alteration of the immune response during the acute phase of measles versus the recovery phase provides some insight into mechanisms that may contribute to immune suppression. During acute measles, there is a strong Th1 response associated with a rise in IFNγ and IL-2. After resolution of the rash, levels of IL-4, IL-10 and IL-13 are elevated suggesting that Th2 and regulatory T cells are preferentially activated during the recovery phase [57, 79, 80]. IL-12 is produced by antigen presenting cells and promotes Th1 development and production of IFNγ. Therefore, it has been suggested that the lack of IL-12 production during measles recovery may contribute to the Th2 skewing of the cytokine profile and immunosuppression [81, 82]. However, vaccination of monkeys with a recombinant MeV expressing IL-12 resulted in alteration of the Th2 bias of the immune response, had no effect on lymphopenia, and did not improve *in vitro* lymphoproliferative responses [83] Therefore, if IL-12 does play a role in MeV-induced immune suppression it may not be a key player. Th2 cytokine predominance may be important for the development and maturation of the humoral immune response; however, it may also function in preventing the induction of Th1 responses that may be required to combat new infections.

Observations from rhesus and cynomolgus macaques infected with recombinant MeV strains expressing enhanced GFP (EGFP) have provided an additional *in vivo* tool for time-course analysis of infection and development of an alternative model to explain

measles immune suppression [84, 85]. Data from these studies showed that MeV targets lymphoid tissues, preferentially infects memory T cells, and causes lymphocyte depletion and exhaustion in lymphoid tissues during the acute phase of infection. Based on these observations, it has been proposed that preferential infection and subsequent immune-mediated depletion of CD150+ memory T cells results in temporary immunological amnesia [20, 69]. Additionally, the short duration of lymphopenia followed by prolonged immune suppression may be due the expansion of new MeV-specific and bystander lymphocytes that replace depleted memory T cell populations and eventual restoration of immunological memory. Together this model would explain why measles-associated immune suppression extends beyond the period of lymphopenia with increased susceptibility to secondary infections during this time.

1.6 Measles Studies in the Macaque Model

Measles is generally recognized as a human disease. At the beginning of the twentieth century, it was demonstrated that MeV could be transmitted from humans to macaques. Subsequently, numerous studies demonstrated that macaques experimentally infected with wild-type strains of MeV mimic many of the clinical, pathological, and immunological patterns seen in humans [86-88]. As a result, much of our more detailed understanding of measles pathogenesis, immune responses, and sites of virus replication come from studies in nonhuman primates (NHP).

The two NHP species commonly used to study measles pathogenesis are rhesus macaques (*Macaca mulatta*) and cynomolgus macaques (*Macaca fascicularis*). However, clinical signs such as rash and conjunctivitis tend to be more prominent in rhesus

macaques [86, 87, 89]. Marmosets are highly susceptible to measles infection, but have not been studied extensively because unlike humans, they often develop disease that is fatal [90].

Naturally acquired infection with WT strains of MeV tends to cause recognizable, clinical evidence of measles however, many laboratory strains of MeV often do not produce disease in primates. Infections with MeV isolated in cell culture demonstrated the importance of passage history as virus strains passaged in lymphoid cells retained pathogenicity in macaques, and passage in other cell lines often resulted in virus attenuation. B95a cells (EBV transformed marmoset B cell line), PBMCs from human cord blood, or COBLa cells (T cell line from cord blood) are ideal for the isolation of WT MeV from patient samples to retain its pathogenicity in macaques [87, 88, 91]. Alternatively, Bankamp et al. (2008) demonstrated that WT MeV isolated from Vero cells expressing CD150 (Vero/hSLAM) and not in unmodified Vero cells retained its pathogenicity in macaques and induced skin rash [92], thus, demonstrating that CD150 expression of cells used for virus isolation is crucial and that cells do not have to be of lymphoid origin.

Monkeys infected with Bilthoven WT strain of MeV develop Koplik's spots, conjunctivitis, and maculopapular rash by 7-10 dpi. The rash may persist for up to 5 days. Chicago-1, Edmonston wild type, Moraten, STL-91, and LA-89 infection of monkeys typically do not result in clinical illness [87]. MeV infection induces strong specific humoral and cellular responses in macaques and recovery is accompanied by lifelong immunity. The macaque model has been crucial for studying the early events following

virus transmission, especially because in humans measles is typically not recognized before the onset of rash.

1.7 Prevention and Control

Measles resulted in millions of deaths before the introduction of an effective vaccine. The first attempt to vaccinate occurred in 1749 when the Scottish physician, Francis Home, inoculated individuals with blood taken from measles patients. In 1954, Enders and Peebles successfully isolated measles virus from a 13-year old measles patient, David Edmonston. The Edmonston strain of MeV was successfully propagated in human and monkey cells which led to the development of both inactivated and live-attenuated virus vaccines (Figure 1-5) [93]. The inactivated vaccine, licensed in 1963, only provided short-term protection and recipients who were re-infected often developed more severe disease described as atypical measles. However, further attenuation of the live-attenuated virus vaccine has led to the highly effective vaccines used worldwide today [94]. The Schwarz vaccine, which was licensed in 1965, is the standard vaccine in much of the world (Figure 1-5). Maurice Hilleman developed the Moraten strain, licensed in 1968, and it is the only vaccine administered in the United States (Figure 1-5) [95]. Despite the differences in passage history and attenuation methods used to develop the Schwartz and Moraten vaccine strains, they are identical in sequence [96].

The recommended age for vaccination is typically between 9 to 15 months and varies regionally based on optimum age for seroconversion and the probability of acquiring measles before that age. Two doses of the measles vaccine are needed to provide sufficient population immunity to interrupt transmission [97]. The first dose is typically

administered through the primary health-care system at age 9 months in areas where measles remains prevalent and at 12-15 months in low-risk areas. Two strategies in place to administer the second dose are either through the primary health care system or mass vaccination campaigns. These immunization strategies have made remarkable progress in reducing measles incidence and mortality worldwide. However, there are still major logistical, financial, and political challenges that could hinder sustained vaccination efforts important for measles control.

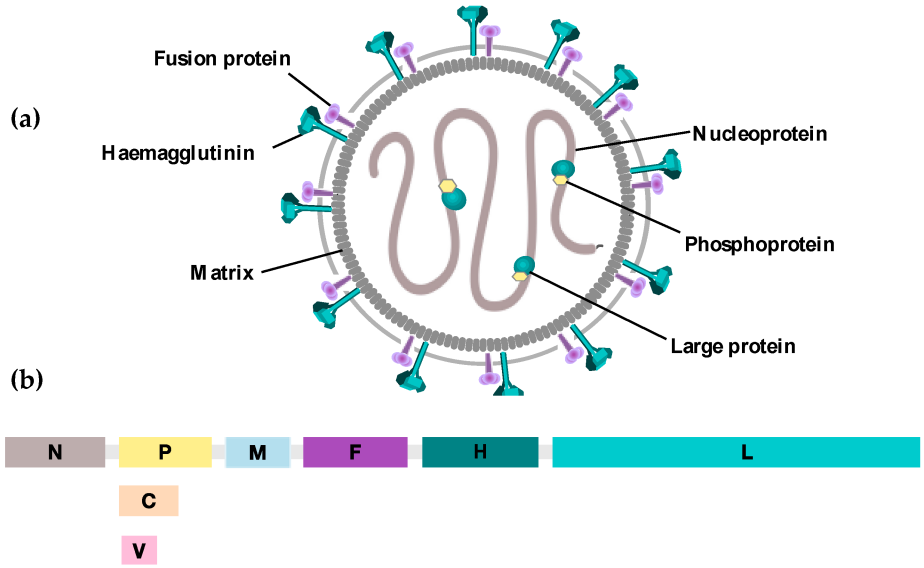


Figure 1-1. Measles virus. Schematic representation of the measles virion structure (A). Measles virus is an enveloped, negative strand RNA virus. The measles virus genome (B) is approximately 16kb and encodes for six structural proteins and two non-structural proteins, C and V proteins. Figure from [98].

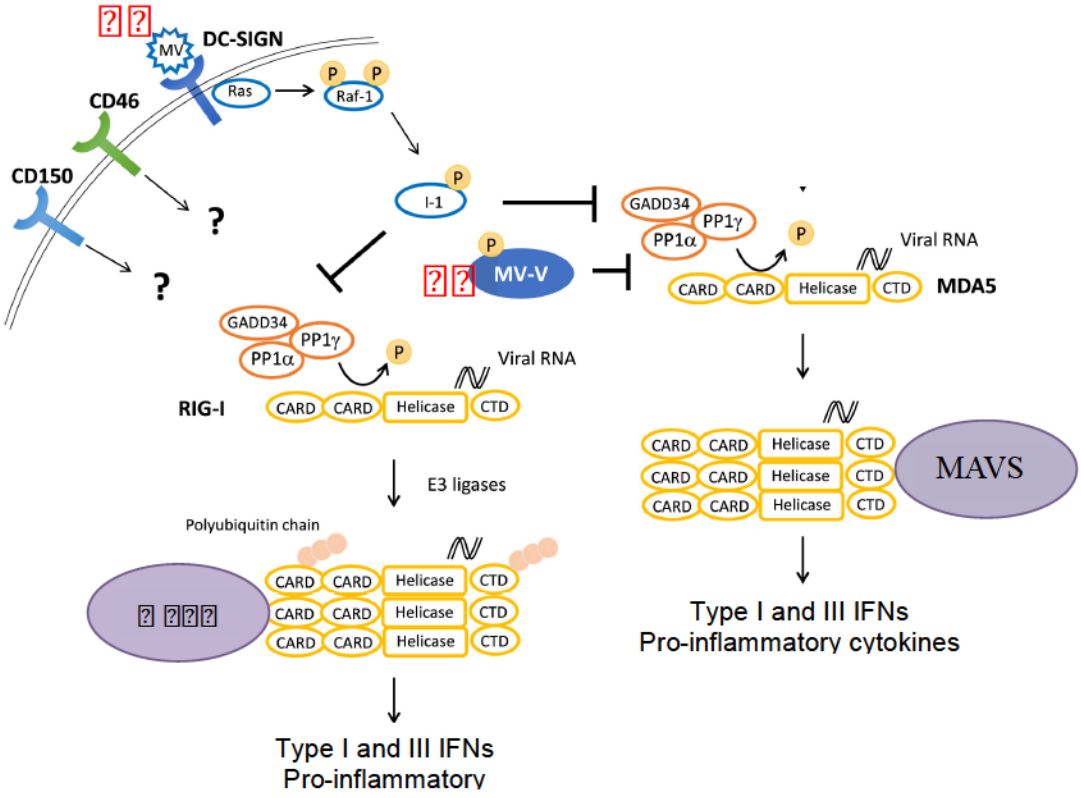


Figure 1-2. Measles virus suppresses activation of RLRs. In response to various RNA viral infections, PP1 dephosphorylates MDA-5 and RIG-I resulting in their activation and the production of type I and III interferons (IFNs). MeV can inhibit this antiviral response by targeting PP1. MeV inhibits PP1 activity in dendritic cells via its interaction with DC-SIGN on the cell surface. MeV binding to DC-SIGN signals the activation of Raf-1, which facilitates the formation of a GADD34-PP1 holoenzyme, thereby inhibiting PP1 phosphatase activity (1). Additionally, MeV V protein blocks PP1 activity by directly binding to MDA-5 and acting as a competitive inhibitor (2) Figure adapted from [99] .

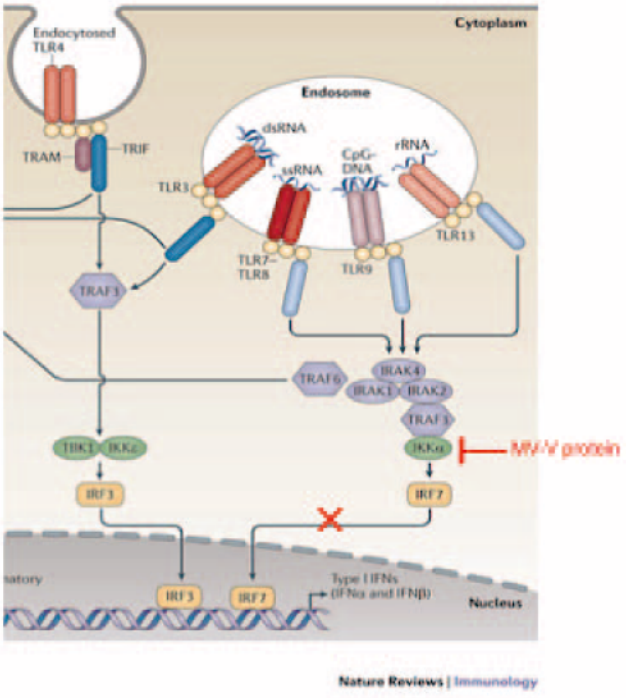


Figure 1-3. MeV can inhibit type I IFN production by TLR7 and TLR9. Activation of TLR7 and 9 results in the recruitment of MyD88, which in turn recruits a complex containing TRAF3, TRAF6, IRAK and leads to the activation of IKKα. IKKα phosphorylates IRF-7 resulting in its nuclear translocation and ultimately the production of type I IFNs. The V protein of MeV directly binds to IKKα preventing the phosphorylation of IRF-7 resulting in the inhibition of type I IFN production. Figure adapted from Nature Reviews Immunology [100].

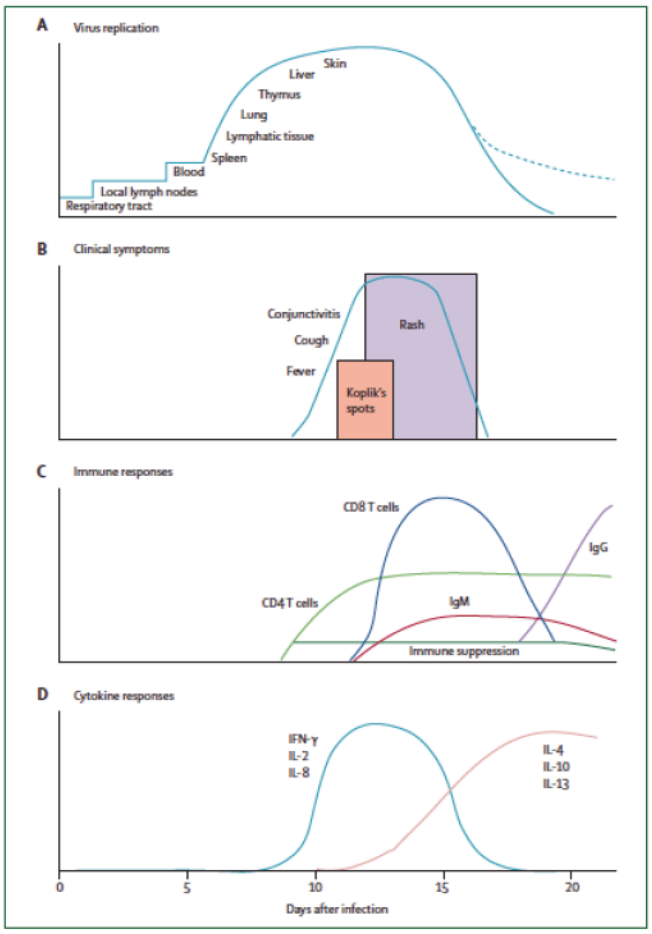


Figure 1-4. Pathogenesis of measles virus infection. Measles virus infection is initiated in the respiratory tract then spreads to multiple organs (A). Clinical symptoms begin 10 to 12 days post-exposure and the appearance of rash begins with the clearance of infectous virus (B). The rash is a manifestation of the adaptive immune response. There is a robust virus-specific immune response during infection, including rapid expansion of T cells and prolonged production of IgG (C). Cytokine profiles include the predominant production of the type I cytokines, IL-2 and IFNγ, followed by a switch to type II and regulatory T cell cytokine production (D). Figure from [22]

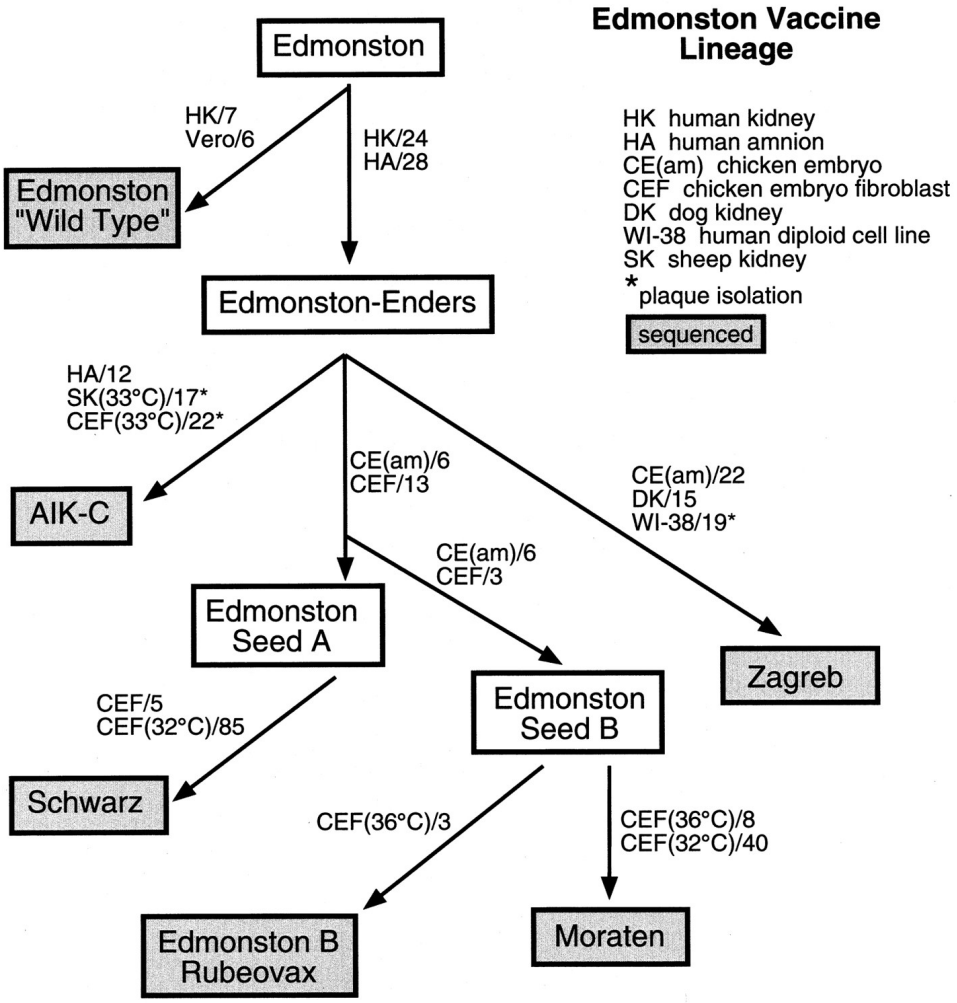


Figure 1-5. Passage history of Edmonston vaccines. Several MeV vaccines were derived from a single clinical isolate called the Edmonston strain. Enders et al. developed the first MeV vaccine by serial passage of the virus isolate in human kidney cells. The virus was attenuated by subsequent passage in chicken embryos and chick fibroblast cells. Variations of the Enders approach have led to the development of a number of Edmonston-based vaccines. Figure from [101]

Chapter 2

Virus clearance and the innate immune response during a primary wild-type measles infection

2.1 ABSTRACT

Measles remains a significant childhood disease. In most individuals, the immune response is able to successfully clear measles virus (MeV) infection and life-long immunity is established. However, infection is also associated with several weeks of immune suppression. As a result, most measles-associated deaths are attributed to secondary infections. Previous work has shown that viral RNA persists in PBMCs four to five times longer than infectious virus. The goal of our study was to further characterize the prolonged presence of viral RNA in multiple tissues and also in various immune cells using a rhesus macaque model. We found that viral RNA could be detected in the blood and lymph node months after infectious virus is cleared, and preferentially persists in B cells and monocytes.

The innate immune response to viral infection often includes induction of type I IFNs and production of antiviral proteins. A number of *in vitro* and *in vivo* studies have shown that neither wild-type nor vaccine strains of MeV induce type I IFN. To gain more insight into MeV inhibition of type I IFN induction, we assessed levels of biologically active IFN by bioassay and used a PCR array to assess the differential expression of genes involved in type I IFN production. Our work confirms previous observations that type I IFN induction is inhibited by MeV infection and suggests that MeV modulates the activation of various pattern recognition receptors (PRRs) involved in the type I IFN pathway.

2.2 INTRODUCTION

Measles virus is a member of the *Paramyxoviridae* family and has a negative-sense, single-stranded RNA genome measuring approximately 16 kb that encodes eight proteins. The hemagglutinin (H) and fusion (F) surface glycoproteins mediate virus fusion and entry. The matrix (M) protein lines the interior of the viral envelope. The nucleocapsid (N) protein is the first to be transcribed from the genome and is therefore, the most abundant of the viral proteins [1]. The genomic RNA is wrapped within the N protein and is packed within the envelope with the phosphoprotein (P) and large polymerase (L) protein attached [1, 102]. There are two non-structural proteins, C and V, encoded within the P gene that interact with cellular proteins and modulate host immune responses [103-105].

Measles is an important cause of childhood morbidity and mortality. Measles results in systemic illness and is associated with persistent viral RNA and several weeks of immune suppression. Paradoxically, measles infection also induces robust virus-specific responses leading to the eventual viral clearance and the establishment of life-long immunity. Activation of the innate immune response is essential for the initial control of viral infections because it slows down replication before adaptive immunity becomes activated. Like many other viruses, measles has acquired various strategies to evade the host immune response including, modulation of IFN responses.

Prolonged clearance of MeV RNA

Measles is a systemic disease transmitted via the respiratory route. Epidemiological evidence suggests that persons with measles are infectious several days before and after

the onset of rash [106]. Clinical recovery occurs soon after the onset of rash at 10-14 days post-infection and coincides with the clearance of infectious virus from the blood. Despite the rapid clearance of infectious virus, MeV RNA persists in peripheral blood mononuclear cells (PBMCs), urine and nasopharyngeal secretions in naturally infected children for several months [107, 108]. Mechanisms of immune-mediated clearance of infectious virus and viral RNA from different types of cells are still unknown, but likely to vary and occur at different rates. However, T-cell primed macaques cleared viral RNA more rapidly than unimmunized animals after challenge with wild-type MeV [109]. In addition, MeV RNA can still be detected in HIV-infected children one-month after hospital discharge [107]. In monkeys, depletion of CD8+ T cells, but not B cells results in a higher and more prolonged viremia [49]. These data suggest that viral clearance is delayed when cellular immune responses are compromised.

Subsequent studies in experimentally infected rhesus macaques characterized the dynamics of MeV RNA clearance from PBMCs [23]. These studies demonstrated that viral RNA clearance from the peripheral circulation occurs in three phases. After an initial peak in viral RNA at 7-10 days, there is a rapid decline coincident with the clearance of infectious virus (10-14 days), followed by a rebound phase with up to a 10-fold increase in RNA levels (14-24 days) and then a slow decline to undetectable levels by 60 days post-infection [23]. However, viral RNA can still be detected in lymph nodes, and potentially other tissues, at later time points.

Regulation of type I interferon production by measles

The type I interferon (IFN) family includes IFNα (13 subtypes in humans) and IFNβ. Almost all cells in the body can produce IFNα/β, and this usually occurs via the stimulation of pattern recognition receptors (PRRs) by microbial products. The RIG-I-like receptors (RLR), retinoic acid-inducible gene (RIG-I) and melanoma differentiation-associated gene (MDA-5) are the main cytosolic PRRs that sense viral RNA. Activation of RIG-I and MDA-5 result in a cascade of downstream signaling events, which lead to the induction of various transcription factors that activate the transcription of IFNα/β. In most cases, the transcription factors IFN-regulatory factor 3 (IRF3), IFN-regulatory factor 7 (IRF7), nuclear factor-κB (NF-κB), and activator protein 1 (AP-1) are the primary regulators of IFNα/β transcription. A number of paramyxoviruses, including measles virus, have the ability to regulate the production of type I IFN. The measles genome encodes for two non-structural proteins, C and V, within the P gene that interact with cellular proteins and antagonize type I IFN induction [103-105]

Measles virus has evolved multiple strategies to inhibit RLR sensing and activation. Recent studies suggest that measles virus inhibits type I IFN production by targeting PP1 phosphatases thereby preventing RIG-I and MDA-5 dephosphorylation and activation [37, 39] The MeV V protein blocks IFN induction by directly binding to MDA-5 [36] or by a forming a complex with PP1α/γ ultimately preventing MDA-5 and RIG-I dephosphorylation and activation (Chapter 1, Figure 1-2) [37, 38]. Additionally, MeV can target PP1 in dendritic cells (DCs) by interacting with DC-SIGN leading to the suppression of RIG-I and MDA-5 activation (Chapter 1, Figure 1-2) [39]

Alternatively, the V protein of MeV can prevent type I IFN induction by TLR7 and TLR9 by directly binding to IKKα or IRF-7 [36, 110, 111]. The MeV C protein has a more indirect effect on type I IFN induction. The C protein is a negative regulator of viral RNA synthesis and thus acts to inhibit IFN induction via blocking the production of PAMPs [8].

The transcription factor, NFkB, is involved in the regulation and efficiency of IFNβ transcription along with the upregulation of a large number of genes that encode for proinflammatory cytokines, chemokines, cell survival genes, and antimicrobial proteins. In vitro studies have shown that MeV RNA or proteins interact with PRRs and can activate NFkB and IRF-3 signaling pathways in a strain-dependent and cell-type specific manner. Epithelial cells show activation of NFκB and AP-1 (activator protein-1), however their activation is suppressed in acute and persistently infected monocytes [25-28]. Moreover, the V protein binds the NFκB subunit p65 (RelA) [104]. This interaction prevents NFκB nuclear translocation, thereby interfering with IFNβ transcription.

Regulation of type I interferon signaling by measles virus

The measles virus V protein interferes with type I IFN signaling. IFNα and IFNβ signal through direct binding to the transmembrane cell surface receptors, IFNAR1 and IFNAR2. Ligation of IFNAR results in activation and recruitment of Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2). In the canonical pathway, activated JAK1 and TYK2 phosphorylate signal transducer and activator of transcription 1 (STAT1) and STAT2. Phosphorylation of this complex leads to its nuclear translocation and ultimately the induction of interferon-stimulated genes (ISGs). Yeast two-hybrid screens and co-

immunoprecipitation studies have shown that the measles virus V protein binds directly to STAT1 and JAK1 to block type I IFN signaling [112]. The V protein also prevents the phosphorylation of Tyk2 [112].

The magnitude of MeV RNA persistence in various tissues past 90 dpi have not yet been investigated, and the mechanism and consequences of persistence are not clear. Additionally, it is clear that the innate response to MeV does not involve the induction of type I IFN however, changes in the gene expression profile associated with type I IFN activation have not yet been described. Therefore, the overall goals of our study were two-fold. First, to determine the extent of MeV RNA persistence in multiple tissues and various subsets of immune cells through 6 months post-infection; and second, to assess type I IFN activity and alterations in the expression of genes associated with type I IFN activation and signaling during MeV infection. We hypothesize that a primary wild-type measles infection will be characterized by the prolonged presence of MeV RNA in PBMCs of the blood and lymph node with preferential persistence of viral RNA in B cell populations. In addition, we expect that MeV infection will result in the inhibition of type I IFN activity along with a down-regulation of genes associated with type I IFN production and activation.

2.3 MATERIALS AND METHODS

Animals, infection and procedures

Six 3-year-old male measles-naïve rhesus macaques (*Macaca mulatta*; 14Y, 17Y, 24Y, 31Y, 46Y and 50Y) were obtained from the Johns Hopkins Primate Breeding Facility. Samples from a second group of rhesus macaques (46U, 55U, 67U, 40V, 43V, and 55V)

from a previous study were also used in the current study [23]. The Bilthoven strain of wild-type MeV (genotype C2; gift of Albert Osterhaus, Erasmus University, Rotterdam) was grown in phytohemagglutinin-stimulated human cord blood cells and assayed by plaque formation on Vero/hSLAM cells [113]. Following baseline measurements, monkeys were infected intratracheally with 10^4 plaque-forming units of MeV in 1 ml PBS. Upon development of a rash, group 1 monkeys received either two daily doses of vitamin A (100,000 units, Vitamin Angels, Santa Barbara, CA; 14Y, 24Y, 50Y) or placebo (17Y, 31Y, 46Y). No differences were observed between supplemented and non-supplemented macaques, so data have been pooled. Macaque 24Y did not develop measles-specific antibodies so he was excluded from all data analysis, and will be discussed further in Chapter 5.

Heparinized blood was collected from the femoral vein before infection and every 3-14 days after infection for six months. PBMCs and plasma were isolated by whole blood gradient centrifugation on Lympholyte-Mammal (Cedarlane Labs). Inguinal lymph node biopsies were collected at days 71 and 154 post-infection. For all procedures, monkeys were sedated with 10-15 mg/kg ketamine intramuscularly. All studies were performed in accordance with experimental protocols approved by the Johns Hopkins University Institutional Animal Care and Use Committee.

Viremia

Infectious MeV in blood was measured by co-cultivating serially diluted fresh PBMCs (10^5 to 10^0) with Vero/hSLAM cells in 48-well plates. Co-cultures were incubated for 5-6 days at 37°C, 5% CO_2. Cytopathic effects in each well were assessed

and the 50% tissue culture infectious dose ($TCID_{50}$) calculated. Level of viremia is expressed as the log$TCID_{50}$ per million PBMCs.

RT-PCR

To monitor MeV shedding from the respiratory tract, nasal secretions were collected from both nostrils with sterile cotton swabs and immersed in PBS. Samples were centrifuged to collect the cells and supernatant fluids for storage at -80°C. The RNeasy® Plus Micro Kit (Qiagen) was used to isolate RNA from the nasal cells. RNA was eluted in 30µl of RNase-free water and 10 ng or 10 µl were used for RT-PCR. Primers MV41 and MV42 were used to amplify a 350 base pair N gene sequence (Table 2-1), and human β-actin RT-PCR primers (Agilent) were used as a control for RNA quality. PCR products were run on gels and read as positive or negative.

MeV N Gene Expression – qRT-PCR

MeV RNA in PBMCs and mononuclear cells isolated from the LN was quantified by qRT-PCR as previously described [23, 114]. Mononuclear cells isolated from the LN were first sorted using magnetic beads into CD3+, CD20+, and CD14+ populations. Briefly, total RNA was isolated from $2x10^6$ PBMCs and the N gene was amplified (Applied Biosystems Prism 7700) using a one-step RT-PCR kit with TaqMan primers and probe (Table 2-1). Controls included GAPDH amplification (Applied Biosystems) and RNA isolated from cultured PBMCs from MeV-naïve monkeys. Copy number was determined by construction of a standard curve from 10^1-10^8 copies of RNA synthesized by in vitro transcription from a plasmid encoding the Edmonston N gene. The sensitivity

of the assay was 50-100 copies. Data were normalized to the GAPDH control and expressed as [(copies of MeV N RNA)/(copies of GAPDH RNA)] x 5,000.

MeV N Gene Expression – ddPCR

Digital droplet PCR (ddPCR) was used to quantify MeV RNA in various subsets of immune cells. Isolated PBMCs were sorted using magnetic beads into CD3+, CD20+, and CD14+ populations. The RNeasy® Plus Micro Kit (Qiagen) was used to isolate RNA from sorted cells. RNA was eluted in RNase free water and 100 ng were used to make cDNA using the iScript™ Advanced cDNA synthesis kit (BioRad, Hercules, CA). A 20 µl mixture of primers, probes, Bio-Rad 2X Supermix (no dUTP) and 5 µl of cDNA was prepared and emulsified with droplet generator oil (Bio-Rad, Hercules, CA) using a QX-100 droplet generator (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Samples were multiplexed with primers and probes specific for MeV N gene (5' FAM; Table 2-1) and GAPDH (5' HEX). The droplets were then transferred to a 96 well reaction plate (Eppendorf, Hauppauge, NY) and heat-sealed with pierceable sealing foil sheets (Thermo Fisher Scientific, West Palm Beach, FL).

PCR amplification was performed in the sealed 96 well plate using a GeneAmp 9700 thermocycler (Applied Biosystems, Grand Island, NY) with the following cycling parameters: 10 minutes at 95°C, 40 cycles consisting of a 30 second denaturation at 94°C and a 60 second extension at 59°C, followed by 10 minutes at 98°C and a hold at 12°C. Immediately following PCR amplification, droplets were analyzed using a QX100 droplet reader (Bio-Rad, Hercules, CA), in which droplets from each well are aspirated, streamed toward a detector and aligned for single-file two-color detection. All samples

and controls were run in quadruplets. Data are reported as [(copies of MeV N RNA)/(copies of GAPDH RNA)] x 5,000.

Interferon bioassays

Serum samples collected from eight macaques (14Y, 17Y, 31Y, 46Y, 50Y, 46U, 55U, and 67U) at days 0, 3, and 7 post-infection were analyzed for interferon production by bioassays. Vero cells were grown overnight in 96-well flat-bottom plates (Corning). Vero cells were then incubated with serum samples, recombinant IFNα (PBL Assay Science), or recombinant human IFNβ (Sigma) for 24 hours at 37°C, 5% CO2. IFNα and IFNβ controls were assessed in the range of 0.1-100,000 units/mL. The cells were then challenged with vesicular stomatitis virus expressing green fluorescent protein (VSV-GFP, a gift from Sean Whelan at Harvard Medical School, Boston, Mass.) at an MOI of 1.0 for 24 hours at 37°C, 5% CO_2. As a negative control, Vero cells were incubated with media only and were not challenged with VSV-GFP. The positive control wells were incubated in media only and were challenged with VSV-GFP. Following infection, cells were trypsinized, washed with 1X PBS, and fixed with PBS + 1%FBS +1% formaldehyde for flow cytometry analysis to determine %GFP-positive cells.

PCR Array

The expression of genes associated with the type I interferon response was profiled by Rhesus Macaque Type I Interferon RT^2 Profiler™ PCR Array (SA Biosciences, Qiagen, Valencia, CA) which targets 84 different core genes involved in this pathway. Total RNA was extracted from PBMCs collected from macaque 31Y at days 0

and 7 post-infection and cDNA was synthesized using the RT^2 First Strand kit (SA Biosciences, Qiagen, Valencia, CA). RT-PCR was performed using the RT^2 SYBR Green qPCR Mastermix using Applied Biosystems Prism 7700 as instructed by the manufacturer (SA Biosciences, Qiagen, Valencia, CA). Data were analyzed by the RT^2 Profiler web-based software (SABiosciences) using the $2^{-\Delta\Delta CT}$ method. The expression levels for genes of interest were normalized to β2-microglobulin (B2M) and HPRT1. Based on a set of housekeeping genes on the PCR array, the software identifies the genes with the most stable expression. The C_T values for those genes are then geometrically averaged and used for the $\Delta\Delta C_T$ calculations. DAVID Bioinformatics Database 6.8 (NIAID/NIH) was used for Pathway analysis.

2.4 RESULTS

Slow clearance of MeV RNA from PBMCs and nasal secretions

We have previously shown that viral RNA persists in various tissues months after infectious virus is cleared during a primary MeV infection of young macaques (1-2 years of age) [23]. Because we were using an older cohort of macaques for this study, which offers the advantage of increased sample collection, we first wanted to examine whether age would impact the rate of viral clearance. Infectious virus in the blood was monitored by co-cultivation of PBMCs with Vero/hSLAM cells. All monkeys developed a viremia by day 7 and cleared infectious virus from PBMCs by day 18 (Figure 2-1). Additionally, all macaques developed a rash by 10 days post-infection (dpi) which was cleared by 14 dpi (Figure 2-1).

MeV RNA was detected in respiratory secretions by 7 to 10 days after infection followed by continued shedding for 1-2 weeks (Table 2-2). MeV RNA in PBMCs gradually decreased after clearance of infectious virus and became undetectable 60 to 70 days after infection (Figure 2-2). These data confirm that prolonged presence of viral RNA occurs in three phases and is characteristic of primary MeV infection (Figure 2-2) [23].

MeV RNA detection in various subsets of immune cells

To determine the cell types in which MeV RNA could be detected, we used ddPCR (PBMCs) and qRT-PCR (LN) specific for the MeV nucleoprotein (N) gene (Figure 2-3). Cells isolated from PBMCs and inguinal lymph node biopsies were sorted into B cell, T cell, and monocyte populations using magnetic beads. In PBMCs, MeV RNA preferentially persisted in CD20+ B cells and was detectable before 60 dpi (Figure 2-3a). Viral RNA levels in sorted PBMCs were below the limit of detection after 60 dpi consistent with viral RNA decay in total PBMCs (Figure 2-2). In the lymph node, MeV RNA was detectable only in CD20+ B cells at 71 dpi, but could be detected in CD3+ (T cells), CD20+, and CD14+ (macrophages) cells at 154 dpi (Figure 2-3b).

Detection of type I IFN activity

Interferon bioassays were performed to determine the presence of type I IFN in serum samples collected at day 0, 3 and 7 post-infection. For this assay, the presence of type I IFN is assessed by the ability of cells incubated with the serum to block VSV infection, thereby resulting in a decrease in the percentage of GFP+ cells. There was no

detectable IFN activity at 3 or 7 days after infection (Figure 2-5b). The sensitivity of the assay varied for IFNα and IFNβ (Figure 2-4). The detection limit for IFNα was in the range of 10-100 units/ml. However, titrations of IFNβ indicated that its activity could only be detected at higher quantities, between 10^4 and 10^5 units/ml (Figure 2-4). It is possible that IFNα/β is present in serum samples, but outside the limit of detection. Negative control wells were incubated with media only, were not challenged with VSV-GFP and there were no GFP+ cells detected in these samples. The positive control wells were incubated in media only and challenged with VSV-GFP, these samples were not protected from infection and GFP+ cells were detected at frequencies greater than 90% (Figure 2-5a).

Differential expression of type I IFN associated genes

To begin dissecting the underlying mechanism by which measles virus infection modulates type I IFN activity in PBMCs, we profiled the expression of genes associated with the type I IFN pathway before and at days after infecion using a PCR array. The results showed an increase in expression of genes associated with the RLR and TLR pathways, including TICAM1 (TRIF), TLR3, TLR7, IFNβ, IFNω, ISG15 and IL-6. However, other RLR and TLR pathway associated genes were down-regulated, including TLR8, TLR9, IFIH1 (MDA-5), IRF3, IRF5, and IFNα1. Moreover, there was down-regulation of genes involved in the cytosolic DNA sensing pathway that promote the induction of type I IFN and proinflammatory cytokines, including ADAR1, IRF3, and CASP1.

Lastly, some of the genes that were down-regulated by measles virus infection were associated with viral carcinogenesis or the development of human malignancies, these included SP100, IRF3, MAMU-B (MHCI), and STAT3. The expression of all genes differentially regulated during the acute phase of measles infection, prior to the onset of rash, and their functions are described in Table 2-3 and Table 2-4.

2.5 DISCUSSION

Measles virus RNA persistence

MeV is able to efficiently replicate in cells of the immune system. In this study we have used experimental infection of rhesus macaques to characterize the prolonged presence of MeV RNA during primary infection. We have shown that MeV RNA can be detected in multiple sites months after infectious virus is cleared from the blood. While viral RNA was only detected in PBMCs until approximately 60 dpi, lymphoid tissue harbored viral RNA through six months post-infection. Additionally, we have shown that MeV RNA can be detected in various subsets of immune cells and from various tissues before and after infectious virus has been cleared. In all, we saw that MeV RNA preferentially persists in B cells in PBMCs and B cells and macrophages in the lymph node.

Previous studies have shown that N gene sequences from RNA recovered from five Zambian children 96-109 days after rash onset were identical to the circulating strain, with one child having N- and H- sequences at 118 days post-rash identical to that recovered from the same child during acute illness [108]. Therefore, it is likely that the

prolonged presence of MeV RNA in tissues after apparent recovery is attributable to slow clearance rather than the acquisition of escape mutations. It is possible however, that slow clearance of MeV RNA may be due to its ability to "hide" in certain subsets of immune cells. For example, Epstein-Barr virus (EBV) is able to establish latent infection within its host by persisting in an episomal form in B cells [115-117]. Studies in Zika virus-infected macaques demonstrated that viral RNA persists in lymphoid tissue through at least 35 dpi with preferential tropism for B cells and macrophages [118]. As a result, it has been hypothesized that ZIKV RNA persistence in peripheral tissues may be associated with post-recovery complications such as Guillain-Barre syndrome or other neurological sequelae [118].

Studies from macaques infected with an EGFP-expressing MeV demonstrated that memory T cells were preferentially infected in lymphoid tissues during the acute phase (9-11 dpi) of infection [20]. In the current study, we were able to detect MeV RNA not only in B cells, but also T cells and macrophages in lymphoid tissue at 154 days post-infection (~5 months). Follicular helper T (Tfh) cells support viral replication and are possible latent reservoirs of HIV [119]. Tfh cells have been proposed as the cause of viral "blips" or reactivation in HIV patients who are receiving ART therapy [119]. While further studies need to be done to determine specific T cell subsets that may harbor MeV RNA it is possible that Tfh cells may serve as the primary T cell reservoir.

In all, we saw that a primary wild-type measles infection resulted in the prolonged presence of viral RNA in the blood and lymph node. The consequences of RNA persistence vary depending on viral infection, tissue tropism and target cells. Further studies are needed to determine the exact consequences of the prolonged presence of

MeV RNA in peripheral tissues. The length of time required for clearance from lymphoid and other tissues is not known. However, the continued presence of MeV RNA in lymphoid tissue may explain the immune suppression associated with measles however, it is much more likely to aid in the maturation of virus specific immune responses and development of life-long protective immunity.

Measles infection and type I interferon

MeV has acquired several mechanisms to escape immune surveillance and inhibit attempts by the host immune system to suppress viral replication, clear virus and eliminate virus-infected cells. In this study, we have shown that wild-type MeV infection of rhesus macaques alters production of interferons (IFNs) and IFN-mediated signal transduction. Biologically active IFNα/β was not detectable in serum during the acute phase of MeV infection before the onset of rash. Therefore, it is likely that the lack of IFN induction contributes to the systemic spread and long incubation period of measles. Previous *in vitro* and *in vivo* studies suggest that in the absence of defective interfering RNAs, neither wild-type (WT) nor vaccine strains of MeV efficiently induce Type I or III IFN [35, 120]. Thus, the differential regulation of the IFN response is not important for attenuation.

The exact mechanism(s) by which MeV inhibits the induction of the IFN response is still not fully understood. Some studies suggest that MeV is able to block type I IFN production by preventing activation of MDA-5 [37, 38]. In this study, gene expression analysis showed that MeV infection resulted in the down-regulation of MDA-5 and a gene involved in MDA-5 signaling, IRF3. The V protein of MeV has been shown to

prevent type I IFN induction by TLR7 and TLR9 by targeting IKKα [110, 111]. We observed an increase in TLR7 and a decrease in TLR8 and TLR9 mRNA expression. TLR7, 8 and 9 have extremely similar signaling pathways that involve IRF-5 and IRF-7 mediated activation of type I IFNs [121]. We observed a down-regulation in IRF-5 but not IRF-7. It is likely that measles targets downstream effectors of endosomal TLR signaling to prevent type I IFN induction.

Previous work in measles vaccinated and wild-type infected macaques did not observe significant increases in IFNα and IFNβ mRNA levels by qRT-PCR, when compared to controls [35]. We observed an increase in IFNβ and IFNω, but a decrease in IFNα mRNA levels. Despite these differences, levels of biologically active IFN could not be detected in serum by bioassay, suggesting inefficient protein production regardless of mRNA levels measured. A role for MeV infection on IFNω expression has not been described. However, IFNω is a more potent inhibitor of HIV replication than IFNα2, and treatment of cells with IFNω resulted in a significant increase in ISG15 [122]. We also observed an increase in ISG15 expression, confirming previous findings that its expression may be induced by IFNω activation.

Overall, our work confirmed that MeV infection inhibits the induction of type I IFN. Gene expression analysis suggests that MeV blocks type I IFN production through the down-regulation of MDA-5, TLR8, and TLR 9 pathways. This also suggests that any type I IFN that is produced is likely via the activation of the RIG-I or TLR 3 pathway. It will be important to repeat theses studies using samples from additional macaques to confirm our findings. However, our results begin to provide some additional insight into

the mechanisms underlying MeV regulation of type I IFN production and identify potential gene targets for future functional assays.

Table 2-1. PCR primers and probe sequences used for the detection of the measles virus N gene

Assay	Target	Primer and Sequence
RT-PCR	350 bp amplicon of the measles virus N gene	**MV41** 5'-CATTACATCAGGATCCGG-3' **MV42** 5'-GTATTGGTCCGCCTCATC-3'
qRT-PCR ddPCR	Measles virus N protein	**MVNfwd** 5'-GGGTACCATCCTAGCCCAAATT-3' **MVNrev** 5'CGAATCAGCTGCCGTGTCT-3' **Probe** 5'-CTCGCAAAGGCGGTTACGGCC

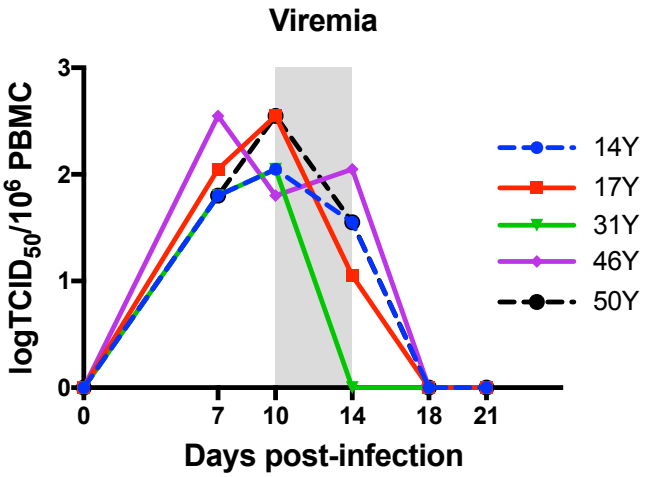


Figure 2-1. Clearance of infectious virus from blood. Macaques were infected intratracheally with the wild-type Bilthoven strain of MeV; viremia was measured by co-cultivation of serially diluted PBMCs on Vero/hSLAM cells. Data are displayed as the log($TCID_{50}$)/10^6 PBMCs. Shaded area indicates period of rash.

	14Y	17Y	31Y	46Y	50Y
Days post infection					
0	-	-	-	-	-
7	-	+	-	-	+
10	+	+	+	+	+
14	+	+	+	+	+
18	-	+	+	-	+
21	-	+	-	-	+
39	-	-	-	-	-

Table 2-2. Detection of MeV RNA in nasal secretions. To monitor MeV shedding from the respiratory tract, nasal secretions were collected from both nostrils with sterile cotton swabs and immersed in PBS. Cells and fluid were separated via centrifugations. RNA was extracted from cell pellets. MeV N gene RNA was measured by RT-PCR and results were read as positive (+) or negative (-) for each monkey.

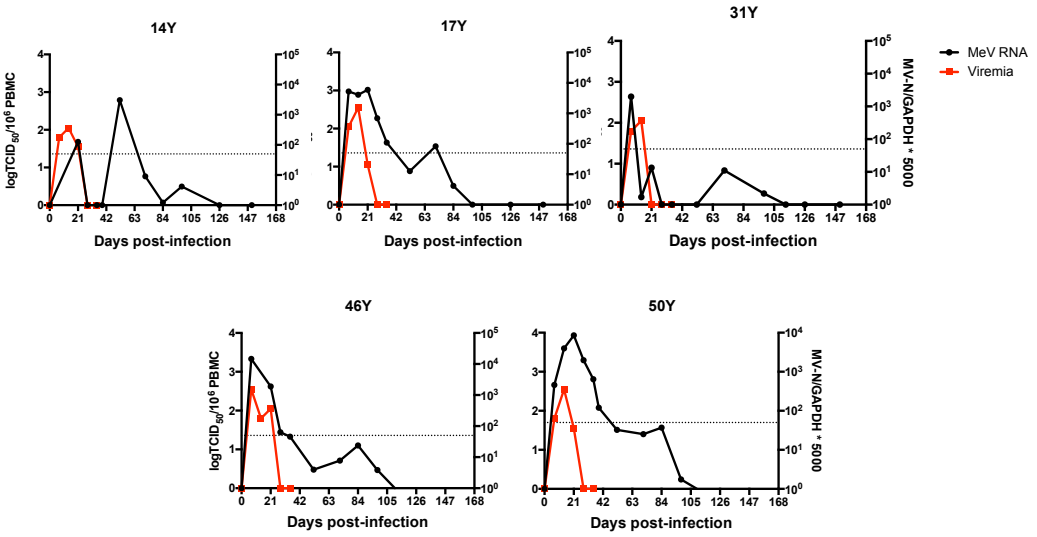


Figure 2-2. Slow clearance of viral RNA from PBMCs. MeV N gene RNA in PBMCs was measured by qRT-PCR. Samples were run in duplicate, means are graphed, with a standard of 10^1-10^8 copies of MeV RNA. MeV RNA load was normalized to the GAPDH control. Results are expressed as [(MeV N RNA copies)/(GAPDH RNA copies)]*5000. The red lines show the titer of infectious virus over time expressed as $logTDIC_{50}$.

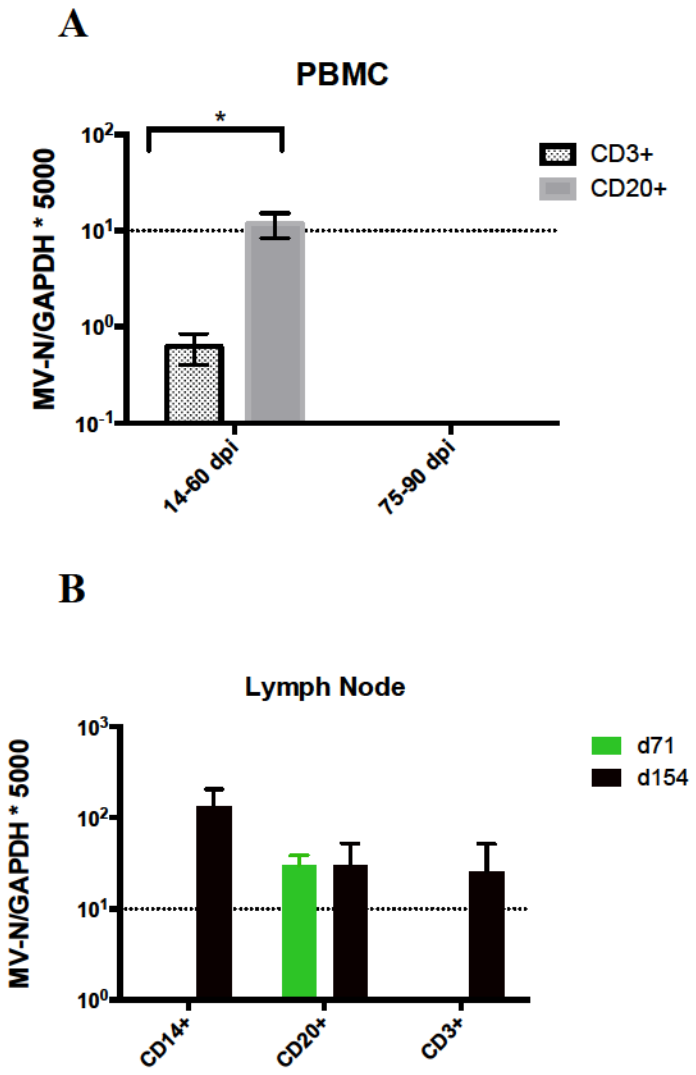


Figure 2-3. Detection of MeV RNA in immune cells. Mononuclear cells from blood (A) and inguinal lymph node (B) were isolated and sorted into CD3+, CD20+, and CD14+ subsets. MeV N gene RNA was measured and data are presented as [copies of MV-N/copies of GAPDH]*5000.

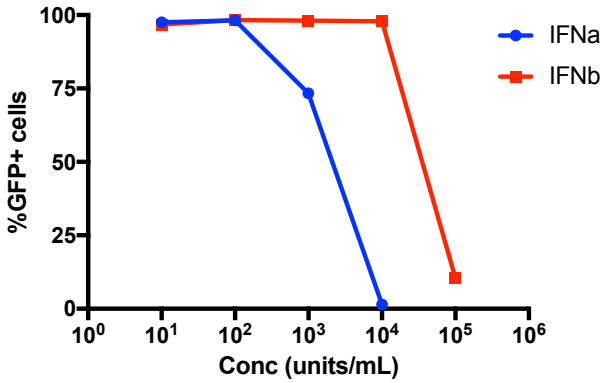


Figure 2-4. Recombinant IFNα and IFNβ detection limits. IFN bioassays were performed and recombinant IFNα and IFNβ were assayed in the concentration range of $10^1 – 10^5$ units/ml. Titrations of IFNs were used to establish the limit of detection for the assay.

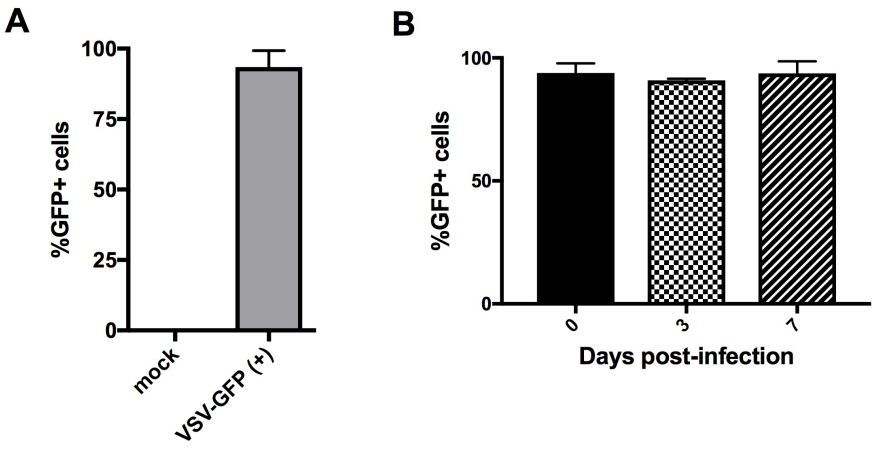


Figure 2-5. Biologically active IFN in serum after MeV infection. Serum collected at days 0 (n=9), 3 (n=3), and 7 (n=9) post-infection were analyzed for type I IFN activity. Vero cells were incubated with media alone (A) or serum (B) and then infected with VSV-GFP. The %GFP+ cells were determined by flow cytometry. Graph shows the mean ±SEM.

Table 2-3. Genes over-expressed at day 7 versus day 0 post-infection

Gene Symbol	Gene Name	Fold Change	Primary Function
TICAM1	Toll-like receptor adaptor molecule 1	74.41	Adaptor protein containing a TIR-domain. TLR3 signaling
IFNW1	Interferon omega-1	28.66	Binds IFNalpha/beta receptor. Type I IFN signaling. Antiviral activity
TLR3	Toll-like receptor 3	25.90	Antiviral. Recognizes dsRNA. Induces activation of NFkB and production of type I IFN
TLR7	Toll-like receptor 7	24.29	Recognizes ssRNA. Induces activation of NFkB, production of type I IFN and pro-inflammatory cytokines.
CRP	C-reactive protein	23.47	Pentraxin-related. Acute phase response to infection and injury.
CYP27B1	Cytochrome P450, family 27, subfamily B, polypeptide 1	22.76	Vitamin D metabolism
IFNB1	Interferon beta-1	18.81	Binds IFNalpha/beta receptor. Type I IFN signaling. Antiviral activity
SLC1A2	Solute carrier family 1, member 2	12.00	Glutamate transporter
HPX	Hemopexin	9.49	Acute phase protein. Oxidative stress.
MAL	Mal, T-cell differentiation protein	7.19	T-cell signal transduction
IL6	Interleukin 6	6.32	Inflammation and B cell maturation
ISG15	Interferon-stimulated gene 15	4.84	Neutrophil chemotaxis. Cell-cell signaling. Antiviral activity.
IFI27	IFN alpha inducible protein 27	4.35	Promotes cell death. IFN-induced apoptosis.
CCL2	Chemokine (C-C motif) ligand 2	4.28	Monocyte and basophil chemotaxis.
GBP3	Guanylate binding protein 3	4.04	Binds guanine nucleotides (i.e. GTP). Antiviral activity.
CAV1	Caveolin 1	3.70	Cell-cycle progression
CIITA	Class II, major histocompatibility complex, transactivator	3.01	Positive regulator of MHCII transcription.
SNCA	Alpha-synuclein-like	2.66	Regulation of dopamine release and transport.

Table 2-4. Genes under-expressed at day 7 versus day 0 post-infection

Gene Symbol	Gene Name	Fold Change	Primary Function
CASP1	Caspase-1	-14.43	Apoptosis. Proteolytic cleavage and activation of IL-1b
PML	Promyelocytic leukemia	-12.60	Associated with PML- nuclear bodies, protein shuttling. Targets STATs, IRFs, & p53 to modulate cellular functions.
VEGFA	Vascular endothelial growth factor A	-8.97	Proliferation and migration of vascular endothelial cells.
TLR8	Toll-like receptor 8	-7.67	Recognizes ssRNA. NFkB activation.Type I IFN production.
TLR9	Toll-like receptor 9	-6.90	Nucleotide sensing. NFkB activation. Production of pro-inflammatory cytokines.
IFIH1(MDA5)	Interferon induced, helicaseC domain 1	-6.23	RNA helicase
IFI35	Interferon-induced protein 35	-6.12	Type I IFN signaling
CDC37	Cell division cycle 37	-4.74	Cell division cycle control
IRF5	Interferon regulatory factor 5	-3.64	Induction of IFNα/β and inflammatory cytokines.
RCBTB1	RCC1, BTB domain containing protein1	-3.64	Cell cycle regulation
IRF1	Interferon regulatory factor 1	-3.63	Activator of IFNα/β transcription. Apoptosis.
LOC719379	Protein kinase C zeta type-like	-3.35	Cell proliferation and differentiation.
MAMU-B	MHC class I antigen	-2.87	Primate specific MHCI antigen**.**
IFNA2	Interferon alpha 2	-2.62	Regulation of IFNα signaling. Antiviral activity.
SLC11A1	Solute carrier family 11, member 1	-2.56	Iron metabolism.
ADAR	Adenosine deaminase, RNA-specific	-2.37	RNA editing by site-specific deamination of adenosines
STAT3	Signal transducer and activator of transcription 3	-2.24	Cell growth and apoptosis. TH17 differentiation.
BST2	Bone marrow stromal cell antigen 2	-2.19	Undetermined.
SP100	SP100 nuclear antigen	-2.14	Tumorigenesis. Cell growth and differentiation.
IRF3	Interferon regulatory factor 3	-2.11	Transcription of IFNα/β and interferon-induced genes.
ISG20	Interferon stimulated exonuclease gene 20	-2.01	Exonuclease that acts on ssRNA.

Chapter 3

Measles-specific humoral immunity: Maturation of antibody responses, germinal center formation and follicular helper T cells

3.1 ABSTRACT

Measles is one of the leading causes of vaccine-preventable childhood morbidity and mortality worldwide. Measle virus infection elicits a robust virus-specific cellular and humoral response that measles leads to the development of life-long protection. The humoral immune response to infection is generally considered most important when assessing vaccine efficacy and protection against measles. The goal of our study was to further understand the development of long-term humoral immunity after measles virus infection using a rhesus macaque model. We show that the antibody response is rapid, specific for various MeV proteins, and sustained for months after infection. However, the maturation of these responses is slow. MeV-specific antibody secreting cells were detected by 3-4 weeks post-infection and sustained through six-months post-infection. In addition, we observed continuous activation of lymph nodes along with Tfh-driven germinal center formation. This suggests that development of MeV-specific humoral responses for at least six-months post-infection may be driven by persistent antigen. Further studies on the development of the humoral response during MeV infection will help define its importance in controlling infection and understanding how long-term immunity is established.

3.2 INTRODUCTION

The host immune response to measles virus is essential for viral clearance, clinical recovery, and the establishment of life-long immunity. During the prodromal phase of MeV infection, early innate immune responses are important for controlling viral replication. Appearance of the rash is associated with the onset of the adaptive immune response. The adaptive immune response to measles consists of virus-specific humoral (antibody) and cellular responses. The humoral immune response to MeV infection is generally considered most important when assessing vaccine efficacy and protection against measles.

Measles is typically accompanied by a rapid and robust antibody response. Antibody is first detectable at the onset of rash. MeV-specific IgM responses appear first, as early as 72 hours post-rash, and are maintained for approximately 28 days [123, 124]. The IgM antibody response serves as a marker of primary infection and is typically absent following reinfection or revaccination. IgG responses appear around 18 days post-infection, increase in amount and avidity overtime, and are presumed to be lifelong. The most abundant and most rapidly produced antibodies are against the MeV N protein, and the absence of N-specific antibodies is one of the most accurate indicators of measles seronegativity [64, 125]. Antibodies to H and F proteins are less abundant, but are important for virus neutralization and protection against measles [43]. The isotypes of MeV-specific antibodies after immunization and natural infection have been well characterized. In general, IgG1, IgG3 and then IgG4 are the predominant isotypes produced [126].

One parameter useful in assessing the quality of an antibody response is antibody avidity. The avidity of an antibody is a measure of the overall strength of the antigen-antibody complex. The strength of antibody avidity increases over time as a result of somatic hypermutaion and affinity maturation of B cells within germinal centers of secondary lymphoid tissues. Measles virus infection or vaccination typically results in the eventual production of high avidity antibodies. Low-avidity antibodies are associated with a poor virus-specific humoral response and have been used to classify primary vaccine failures [127, 128].

Follicular helper T (Tfh) cells are a subset of CD4+ T cells that provide help to B cells and play a key role in the germinal center reaction. Studies have shown that Tfh cells are essential for B cell proliferation, affinity maturation, and the generation of long-lived memory and plasma B cells [129]. Unlike other helper T cell subsets, the differentiation of Tfh cells is a complex and tightly controlled process. A variety of cytokines and transcription factors have been found to aid in each stage of the Tfh development process. The transcription factor, BCL6, is a key master regulator of the differentiation process [129]. Tfh cells are phenotypically recognized by their expression of CXCR5, ICOS, Bcl-6, and PD-1 and functionally by their synthesis of high levels of IL-21 [129].

Previous work has shown that MeV RNA can be detected in the lymph nodes 5-6 months after infection (See Chapter 2). In this study, we characterized the evolution of the MeV-specific humoral immune response in rhesus macaques through six months post-infection. In addition, we investigated changes in lymphoid tissue architecture and the frequencies of Tfh cells in the blood. Our studies suggest that increased proliferation

within lymphoid tissue and circulating Tfh cells promote the prolonged induction and maturation of the MeV-specific humoral response.

3.3 MATERIALS AND METHODS

Animals

Five male rhesus macaques (14Y, 17Y, 31Y, 46Y, and 50Y) infected intratracheally with the wild-type Bilthoven strain of MeV were studied for six months. Heparinized blood was collected from the femoral vein of each animal before infection and every 3-14 days after infection for six months. PBMCs and plasma were isolated by whole blood gradient centrifugation on Lympholyte®-Mammal (Cedarlane Labs).

Plaque Reduction Neutralization (PRN) Assay

Neutralizing antibody concentration in serum was measured using a plaque reduction neutralization assay. The Edmonston strain of MeV was mixed with serially diluted plasma (1:3 – 1:30000) and assayed for plaque formation on Vero cells using a 6-well plate format. Data reported are the reciprocal of the serum dilution at which the number of plaques is reduced by 50%.

Enzyme Immunoassays

EIAs were used to measure MeV-specific IgM and IgG in serum. 96-well Maxisorp plates (Nalgene Nunc International) were coated with lysate from MeV-infected Vero cells (1.16 μg/well; Advanced Biotechnologies). Plates were blocked with 2% non-fat dry milk for 2 hours at 37°C. Serially diluted plasma (1:50 – 1:25600) was added and

incubated at room temperature for 2 hours. Horseradish peroxidase (HRP)- conjugated goat anti-monkey IgM or IgG (Sigma) was used as a secondary antibody. Plates were developed using 3,3',5,5'-Tetramethylbenzidine (TMB) as the substrate and reaction was stopped using 2M H_2SO_4. Plates were read at 450nm. The EIA titer was the highest dilution at which responses were twice the background.

MeV protein-specific IgG responses were measured using H, F, or N antigens. 96-well immunoplates were coated with lysates of L cells-expressing MeV-H or MeV-F, or with baculovirus-generated MeV-N [130, 131]. Antigens were diluted 1:1000 (MeV-H and –F) or 1:2000 (MeV-N). Blocking, serum dilution, IgG detection, and development were performed as above.

To assess the avidity of MeV-specific antibody, EIAs were performed as described above and increasing concentrations (0.5M-3M) of ammonium thiocyanate (NH_4SCN) was added for 15 minutes to disrupt the antigen-antibody interaction following plasma incubation. The avidity index was calculated as the concentration of NH_4SCN required to remove 75% of bound antibody.

Antibody Secreting Cells.

To measure antibody-secreting cells in the peripheral blood and bone marrow, cells were isolated from blood and bone marrow aspirates using density gradient centrifugation with Lympholyte Mammal (Cedarlane Laboratories). Multiscreen HTS HA Opaque plates (Millipore) were coated with MeV-infected Vero cell lysate or with purified goat anti-monkey IgG, IgM, IgA (H&L) (Sigma) and incubated at 4°C overnight. After incubation, plates were washed twice with PBS and blocked with RPMI + 10% FBS at

37°C for 1 hour. Cells (0.5 x 10^6) were added to plates and incubated for 6 hours at 37°C, 5% CO_2. After incubation, plates were washed with PBS-T (1X PBS + 0.05% Tween-20) and bound immunoglobulin was detected with HRP-conjugated goat anti-monkey IgG (1:5000). Plates were developed with stable diaminobenzidine (DAB) solution (Invitrogen) and read on a Immunospot plate reader (Cellular Technology). Samples were run in duplicate and data are presented as antibody-secreting cells (ASCs) per 10^6 PBMCs.

Histology and Immunohistochemistry

Inguinal lymph node biopsies were taken at days 71, 72, 154, or 155 post-infection. Tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Deparaffinized lymph node sections were stained for Ki-67 (1:100; MM1, Novacastra) and using the Masson trichrome method for visualization of collagenous connective tissue fibers. To characterize immune cell distribution within the lymph node, tissue sections were deparaffinized and stained using anti-CD3 (1:400; A052, Dako) and anti-CD20 (1:1000; L26, Dako). Histology and immunohistochemistry staining was performed using an automated Leica® Bond Max system (Leica Biosystems, Buffalo Grove, IL).

Flow Cytometry

Multicolor flow cytometry with intracellular cytokine staining was used to identify MeV-specific circulating follicular T helper cells ($CD4^+$ CXCR5+). PBMCs were stimulated for 12 hours using pooled overlapping H or N peptides (1μg/ml), peptide

diluent dimethyl sulfoxide (DMSO) or staphylococcal enterotoxin B (SEB). Purified mouse anti-human CD28 (BD Biosciences, CD28.2) and anti-human CD49d (BD Biosciences, 9F10) were included with the peptides and DMSO. All stimulation mixes included protein transport inhibitors GolgiStop and GolgiPlug (BD Biosciences).

Live/Dead® Fixable Violet Dead Cell Stain Kit (Invitrogen) was used to gate out dead cells. Prior to surface staining, cells were incubated with a human FcR block (eBioscience). All panels included anti-CD4 (Biolegend, OKT4) and anti-CD3 (BD Bioscience, SP34-2). A "dump gate" was used to gate out cells labeled with anti-human CD14 (BD Biosciences, M5E2) or anti-human CD20 (eBioscience, 2H7). Intracellular staining was done following fixation and permeabilization of cells. The Foxp3 Staining Buffer Set (eBioscience) was used. Intracellular staining was done to detect the transcription factor Bcl-6 (BD Biosciences, Q21-559) and cytokine IL-21 (eBiosciences). Samples were run on a BD FACS Canto II™ flow cytometer and analyzed using BD FACSDiva and FlowJo software.

3.4 RESULTS

Characterization of the MeV-specific humoral response

Enzyme immunoassays were used to assess the dynamics of the MeV-specific antibody response. IgM was detectable by 14 dpi in all macaques, peaked at 21 dpi and was no longer detectable by 28 dpi (Figure 3-1a). Measles virus specific IgG antibodies appeared in circulation 3-4 weeks post-infection and were sustained at elevated levels through six-months post-infection, the last time point tested (Figure 3-1b). To define the specificity of these antibodies we used enzyme immunoassays specific to measles

hemaglutinin (H), nucleocapsid (N), and fusion (F) proteins. All macaques developed antibodies specific to all three measles virus structural proteins. Antibody responses specific for the N protein were highest, followed by H and then F responses (Figure 3-1d). Neutralizing antibodies, measured using a PRN assay, were detectable by 2-3 weeks post-infection and sustained through six months (Figure 3-1c). Because there is growing evidence that the quality of the antibody response is also important for protection we assessed antibody avidity. Maturation of the antibody response was slow, as antibody avidity did not peak until 98-126 dpi (Figure 3-2).

To further characterize the humoral response, we examined the induction of MeV-specific antibody-secreting cells using a B-cell ELISpot assay. MeV-specific antibody-secreting cells began to appear in PBMCs around 14 dpi and peaked later around 50 dpi (Figure 3-3b). In contrast, the maximal number of antibody-secreting cells was around 21 days post-infection in the bone marrow (Figure 3-3d). Measles virus infection resulted in an increased production of total antibody-secreting cells in PBMCs at 14 dpi, while in the bone marrow an increase was not observed until 60 dpi (Figure 3-3a,c). In all, the production of antibody secreting cells is prolonged and maintained in the periphery and bone marrow through six months post-infection.

Germinal center formation in lymph nodes

To determine whether the presence of viral RNA had any impact on lymphoid tissue pathology, immunohistochemistry was performed on tissues collected at 71-72 dpi and 154-155 dpi. Lymph nodes were reactive, with increased cellularity that included plasma cells and macrophages in addition to lymphocytes (Figure 3-4). Proliferation

within the tissues increased over time along with an increase in numbers of germinal centers (Figure 3-4 and 3-5c). At days 71-72 post-infection fewer than 10 germinal centers were identified within a lymph node section of each macaque. By 154-155 dpi, sections from three of the four macaques had almost double the number of germinal centers (Figure 3-5c). The follicles in tissues collected at 154-155 dpi show abnormal germinal centers with marked hyalinization, this was observed in three out of four macaques (Figure 3-5). One macaque was excluded from analysis due to poor tissue quality. Lymph node sections were stained for CD3+ and CD20+ cells to characterize the distribution of T cells and B cells at late time points after infection (Figure 3-6). CD20+ cells were predominantly present around the periphery with fewer B cell areas in deep cortex (Figure 3-6a) while CD3+ cells were scattered within follicles (Figure 3-6b).

CD4+ CXCR5+ cells increase in the blood late in infection

Tfh cells in mice, humans and rhesus macaques express high levels of PD1 in addition to CXCR5, an essential chemokine receptor [132, 133]. While Tfh cells predominantly mediate their function in the lymph node, we sought to characterize these cells in the blood because it is a more easily accessible tissue and several lines of evidence suggest that circulating Tfh cell populations are generated from cells committed to the Tfh lineage and share functional properties with GC Tfh cells [134-136]. We first evaluated the expression of CXCR5 on CD4+ T cells specific for the MeV H- or N-protein (Figure 3-7a). We observed an increase in H-specific CD4+ CXCR5+ T cells from 0.65% ± 0.28 at 39 dpi to 2.61% ± 0.69 by 113 dpi. This same trend was observed with N-specific CD4+ CXCR5+ T cells (0.47% ± 0.09 to 3.97% ± 1.63). Next, we

examined the co-expression of CXCR5 and PD-1 on MeV-specific CD4+ T cells. There was an increase in H- and N-specific CD4+ CXCR5+ PD-1+ T cells over time (Figure 3-7b and d).

IL-21 is highly expressed by Tfh cells and is important for driving plasma cell differentiation [137]. We evaluated the production of IL-21 by CD4+ CXCR5+ T cells. Overall, we observed very little IL-21 production by H- or N- specific CD4+ CXCR5+ T cells. There was one monkey (14Y) that had increased IL-21 production at day 56 post-infection. Also, we have not been able to detect IL-21 in plasma during measles.

3.5 DISCUSSION

In this study, we have shown that the antibody response to measles virus is rapid, however, the maturation of this response is slow. MeV-specific IgM was detectable by 14 dpi, while IgG levels peaked at 21 dpi and were sustained through six months post-infection. These antibodies were neutralizing and specific for the measles virus H, N, and F proteins. The quality of the antibody response gradually increased with peak antibody avidity occurring around 3-4 months post-infection. In addition, we observed the prolonged production of antibody secreting cells in the blood and bone marrow. Together, these data suggest that measles virus infection gradually induces the development of high quality long-lived plasma cells responsible for the long-term maintenance of measles-specific antibodies. Lastly, we observed an increase in proliferation and GC formation in lymphoid tissue, along with increased Tfh cells in circulation as the humoral immune response matured.

The process of affinity maturation arises through a balance between slow but steady elimination of lower affinity clones and variants leading to GC homogeneity and loss of clonal diversity [138]. There is increasing evidence that the avidity of antibodies induced by infection or immunization is important for protection. Vaccination with inactivated measles virus that leads to the rapid production of low avidity antibodies has been associated with primary vaccine failures, and the production of immune-complexes leading to atypical measles upon challenge [139-141]. Avidity maturation is dependent on Tfh cell help to B cells within germinal centers [129, 132, 142-144]. In our study we saw that measles infection resulted in a gradual increase in virus-specific Tfh cells in the blood coincident with an increase in antibody avidity and continued production of antibody secreting cells.

GCs are transient structures that form within secondary lymphoid follicles upon antigen exposure. We have previously shown that viral RNA can be detected in various immune cells within the lymph node at 5-6 months post-infection (Chapter 2, Figure 2-3). In the current study, we have shown an increase in germinal center formation and proliferation in lymphoid tissue at six months compared to 2.5 months post-infection, indicating continued stimulation and antigen exposure. In addition, we observed increased hyalinization within germinal centers. Hyalinization refers to the process by which smooth muscle cells and collagen fibers appear to fuse and take on a homogeneous, acellular, “glassy” appearance [145]. This histological characteristic has been observed in rhadinovirus-infected macaques [146], in HHV-8 (KSHV) positive humans [147], and in chronic SIV and HIV infections [145, 148]. Hyalinization of

reactive germinal centers may be attributed to “burn-out” and is associated with marked lymphocyte depletion and an increase in plasma cells [145, 149].

Emerging evidence suggests additional roles for Tfh cells in virus clearance and as virus reservoirs. Most viral infections are strong inducers of Th1 and Tfh responses, the differentiation of both being induced by IL-12 [129]. LCMV studies in mice suggest that viral persistence and prolonged TCR stimulation promotes the T cell differentiation from Th1 to Tfh cells [129, 150]. In addition, late production of IL-6 is essential for sustaining Tfh cells, promoting GC responses, and clearing infectious virus [129]. In HIV, Tfh cells increase in frequency, serve as viral reservoirs, and combination antiretroviral therapy reduces the proportion of Tfh cells carrying HIV DNA [119, 129]. Our data show that measles infection induces the expansion of Tfh cells and the frequency of these cells was highest later in infection.

In all, a primary wild-type measles infection resulted in the prolonged induction and maturation of the humoral immune response; the dynamics of these responses are summarized in Figure 3-8. These observations correlated with increased proliferation within lymphoid tissue and circulating Tfh cells. Previous observations suggest that MeV RNA persistence in lymphoid tissue may aid in the maturation of virus specific humoral responses (Chapter 2; Figure 3-8). Further studies are needed to determine the distribution and concentration of Tfh cells in lymph nodes, and characterize B cell responses at late phases of measles.

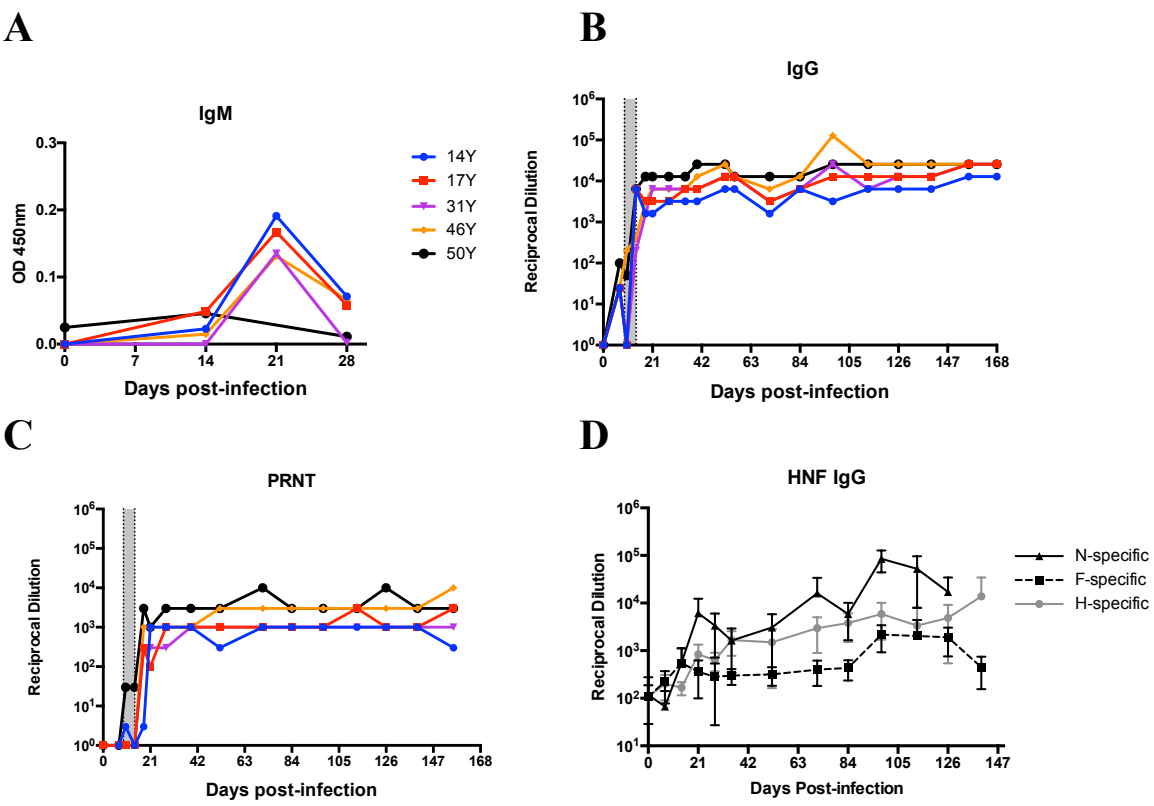


Figure 3-1. Antibody responses in macaques after a primary wild-type MeV infection. Plasma levels of IgM (A) and IgG (B) antibodies from plasma were measured by an EIA using measles virus lysate as antigen. Neutralizing antibody titers were measured using a PRN assay, in which serially diluted plasma collected at various time points was tested for its ability to neutralize the Edmonston strain of MeV in Vero cell (C). Antibodies to H, N and F MeV proteins were measured by EIA, data shown are average responses from five macaques (D).

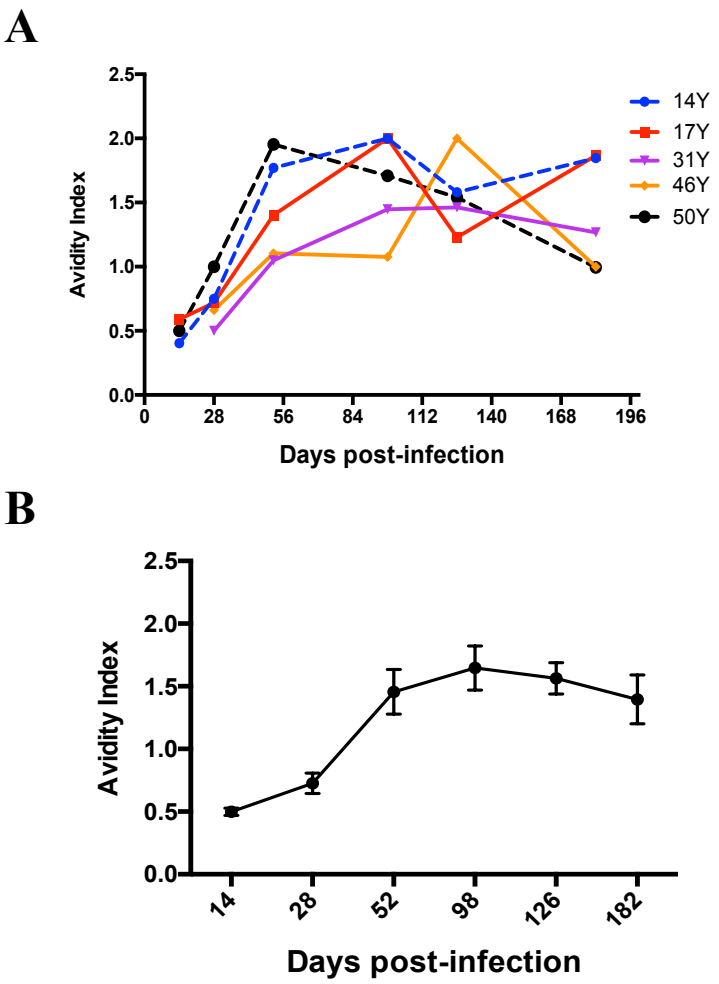


Figure 3-2. Avidity of MeV-specific antibodies. MeV-specific IgG avidity was assessed by determining the minimum concentration of NH4SCN needed to disrupt antigen-antibody interactions. An avidity index (AI) was calculated. Calculation of an AI75 is shown in the graph for each monkey (A) and averaged responses (B), indicating the concentration needed to remove 75% of bound antibody.

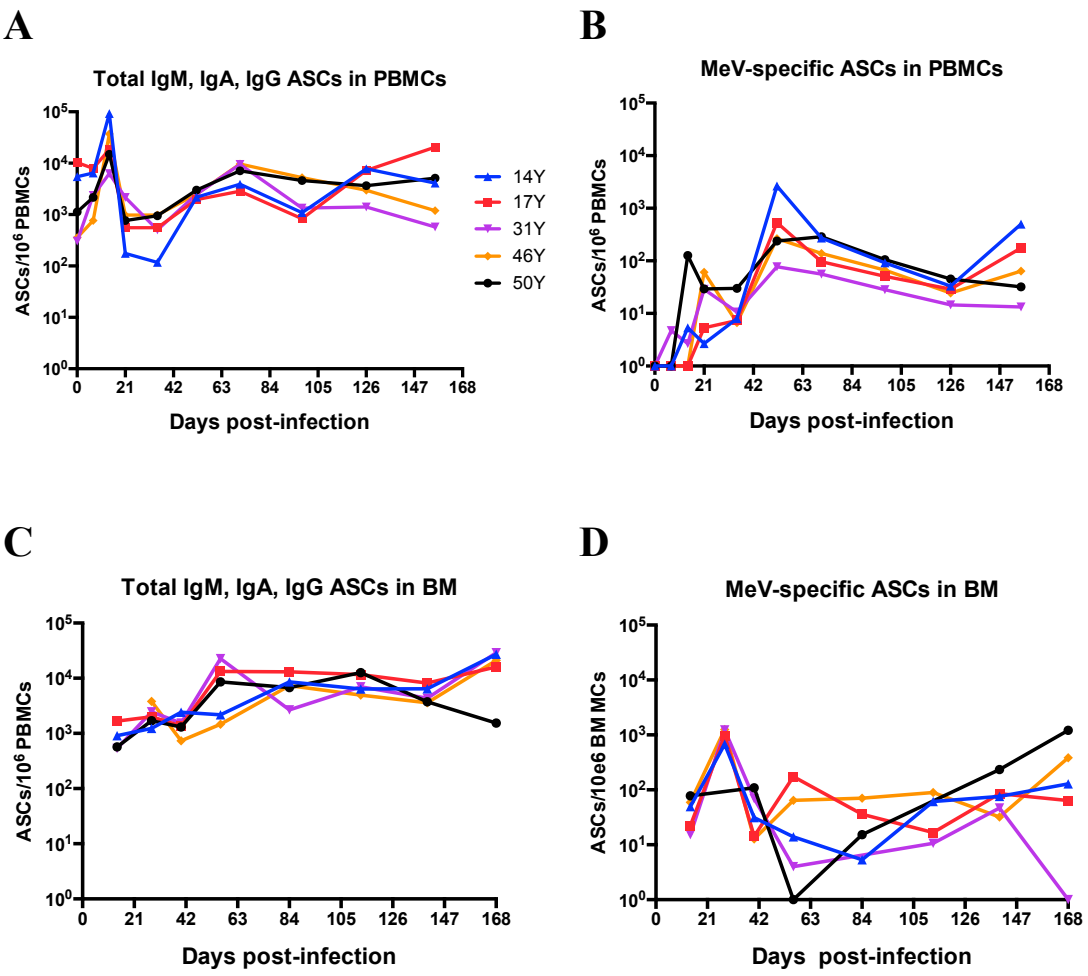


Figure 3-3. Prolonged production of antibody-secreting cells during the course of infection. The humoral immune response was further characterized by examining the presence of total and MV-specific antibody-secreting cells (ASCs) in blood (A and B) or bone marrow (C and D) using a B-cell ELISpot assay. Plates were coated with measles virus lysate or Anti-monkey IgG, IgA, IgM and 5x10^6 cells were added to each well. All samples were run in duplicate and data are plotted are mean ASCs per 10^6 PBMCs.

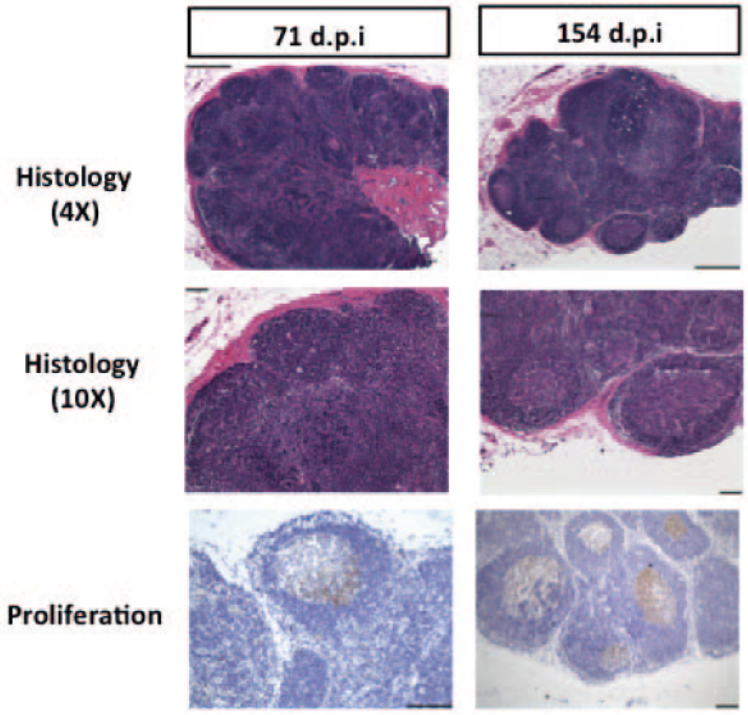


Figure 3-4. Lymph node histopathology. Inguinal lymph node biopsies were collected at 71 dpi and 154 dpi. Tissues were fixed, embedded in paraffin, sectioned, and stained for histological changes (H&E) and cell proliferation (Ki67). Tissues from all five macaques were collected at each time-point and analyzed, panels shown are from monkey 14Y (H&E) and 31Y (Ki67) and representative of the cohort.

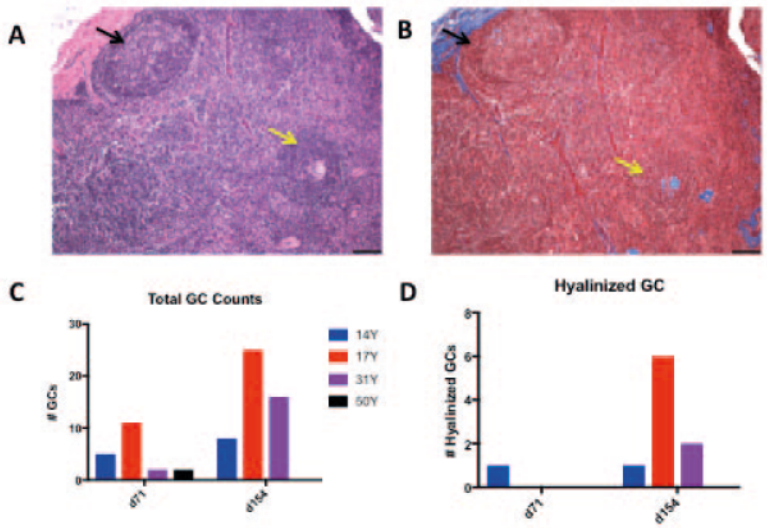


Figure 3-5 Characterization of germinal center formation and structure. Sequential sections of a 154 dpi inguinal lymph node biopsies stained for histological visualization of the basic architecture (A; H&E) and for the content of collagenous connective tissue fibers (B; Mason'sTrichrome stain). Germinal center formation was characterized at 71 (n=4) and 154 dpi (n=3). Normal (black arrow) and hyalinized (yellow arrow) germinal centers for each section were counted for each macaque at each time point. Graphical representation for germinal center counts for each macaque is shown in panels C and D.

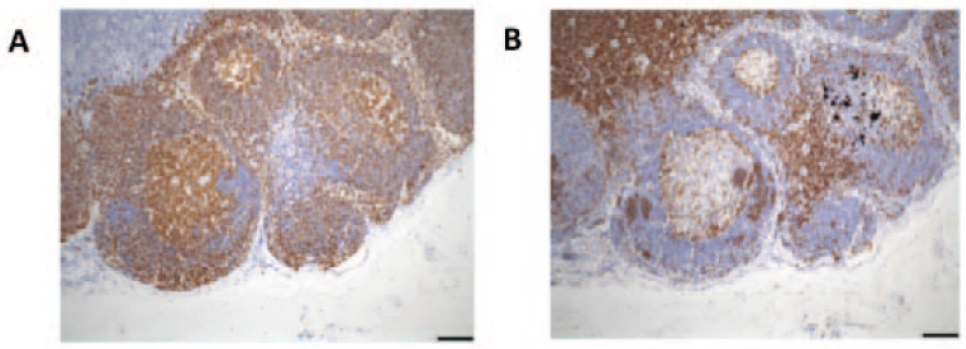


Figure 3-6. Immunohistological profile of CD3- and CD20- expressing cells within lymph node sections. Sequential lymph node sections were stained with anti-CD20 (A) or anti-CD3 (B) to characterize localization of B cells and T cells, respectively, during late phases of measles virus infection in macaques. Images shown are from monkey 31Y at 154 dpi, were photographed at 20X objective, and are representative of all macaques analyzed.

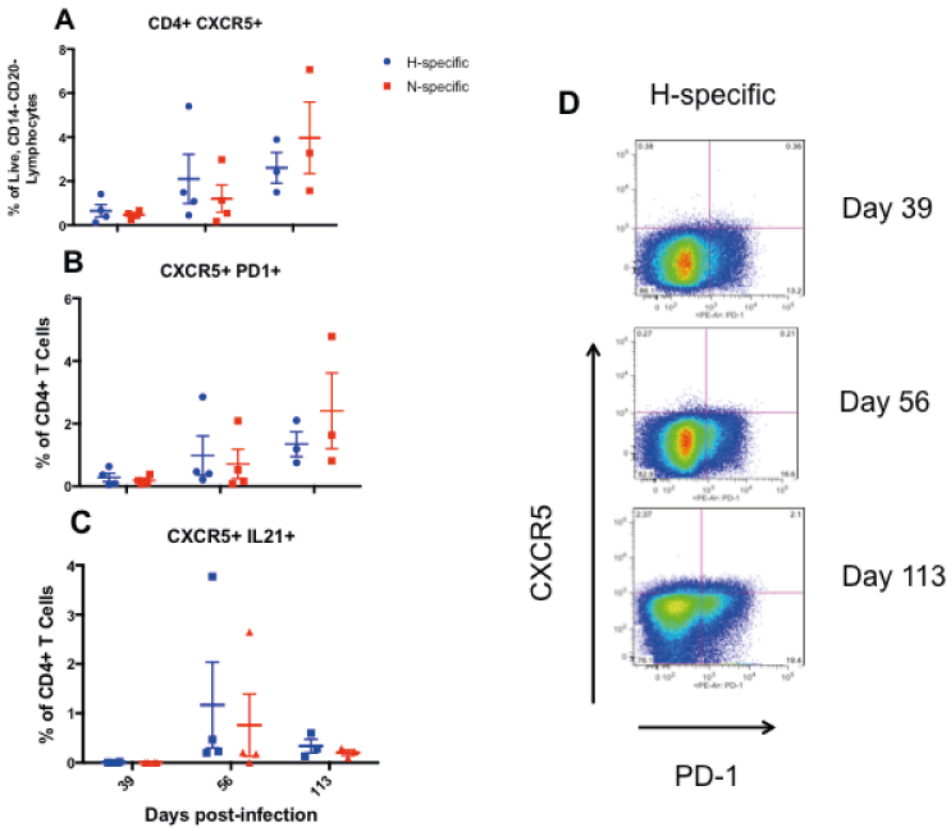


Figure 3-7. Characterization of Tfh cells in the blood. Frequency of H- and N-specific CD4+ CXCR5+ (A), CD4+ CXCR5+PD-1+ (B) and CD4+ CXCR5+IL-21+ T cells in the blood at day 39 (n=4), 56 (n= 4), and 113 (n=3) post-infection. (D) Representative plot showing co-expression of PD-1 and CXCR5 on H-specific CD4+ T cells.

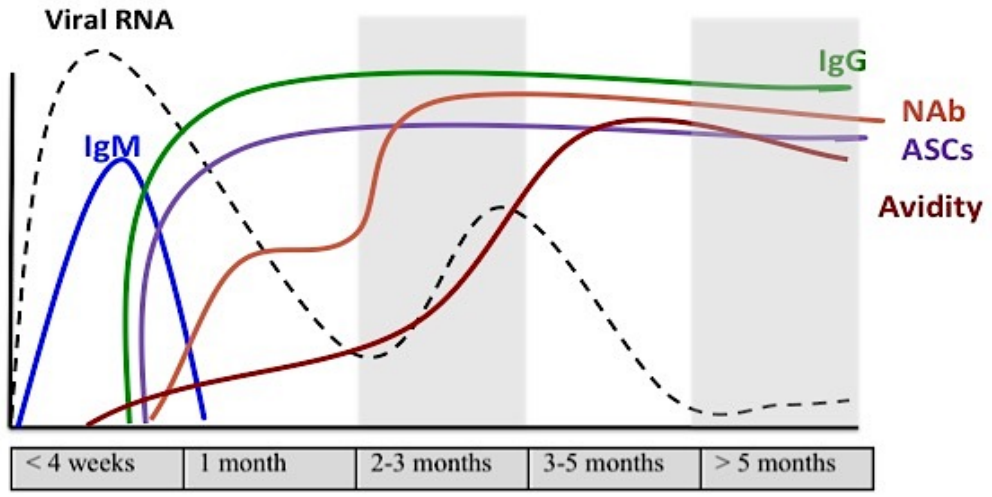


Figure 3-8. A model summarizing the dynamics of the MeV-specific humoral response and viral RNA clearance. Antibody responses to measles virus infection are rapid, IgM (blue line) is detectable between 2-3 weeks post-infection. MeV IgG (green line) responses peak by 3 weeks post-infection, are sustained and specific for various viral proteins (H, N, and F). However, maturation of the antibody is much slower, as high avidity antibodies are produced and peak between 4-5 months post-infection (red line). MeV-specific antibody responses are sustained via the prolonged induction of antibody secreting cells in the periphery and bone marrow (orange line). As MeV-specific humoral responses develop viral RNA (dotted line) in PBMCs is eventually cleared but, can still be detected in the lymph node (shaded areas) between 3 and 5 months post-infection (See Chapter 2).

Chapter 4
The Prolonged Evolution of T Cell Responses during Measles Virus Infection

4.1 ABSTRACT

Measles is an acute viral disease associated both with immune suppression and development of life-long immunity. Clearance of measles virus (MeV) involves rapid elimination of infectious virus during the rash followed by slow elimination of viral RNA. To characterize cellular immune responses during recovery, we analyzed the appearance, specificity and function of MeV-specific T cells between 2 and 24 weeks after respiratory challenge of rhesus macaques with wild type MeV. IFNγ- and IL-17-producing cells specific for the hemagglutinin and nucleocapsid proteins appeared in circulation in multiple waves approximately 2-3, 8 and 18-24 weeks after infection. IFNγ-secreting cells were most abundant early and IL-17-secreting cells late. Both $CD4^+$ and $CD8^+$ T cells were sources of IFNγ and IL-17, and IL-17-producing cells expressed RORγt. Therefore, the cellular immune response evolves during MeV clearance to produce functionally distinct subsets of MeV-specific $CD4^+$ and $CD8^+$ T cells at different times after infection.

4.2 INTRODUCTION

Measles is a highly contagious viral disease that remains an important cause of childhood morbidity and mortality [22] with most deaths due to secondary infections [151, 152]. Measles virus (MeV), the causative agent of measles, is transmitted by the respiratory route and has an incubation period of 10-14 days. From the respiratory tract, MeV spreads to local lymphatic tissue and then to multiple organs including the skin. The prodrome of fever, cough and conjunctivitis is followed by a maculopapular rash associated with development of the adaptive immune response and T cell infiltration into sites of MeV-infected skin cells [140]. Although infectious MeV is cleared soon after the appearance of the rash, MeV RNA persists in peripheral blood mononuclear cells (PBMCs), urine and nasopharyngeal secretions of both naturally infected children [107, 108] and experimentally infected rhesus macaques [23] for several months.

The host adaptive immune response is necessary for control and clearance of virus [153, 154] and both MeV-specific antibody and T cells contribute to gradual clearance of viral RNA from PBMCs [23]. Studies of both humans and monkeys suggest that $CD8^+$ T cells are important for clearance of infectious virus during the rash. MeV-specific cytotoxic T lymphocytes appear in blood during natural infection [50] and experimentally infected macaques depleted of $CD8^+$ T lymphocytes have viremias that are higher and of longer duration than immunologically intact monkeys [49].

Although less well studied, $CD4^+$ T lymphocytes are likely to be essential contributors to a successful immune response to MeV and establishment of life long immunity. Naïve $CD4^+$ T cells develop into functionally distinct subsets defined by the conditions required for differentiation, transcription factor expression and cytokines

produced and important subtypes include Th1 cells producing interferon (IFN)-γ, Th2 cells producing IL-4, Th17 cells producing IL-17 and Treg cells producing IL-10 [155]. Evaluation of cytokines in plasma of children with measles suggests that $CD4^+$ T cells predominantly produce IFNγ during the rash period followed by a later switch to IL-4, IL-10 and IL-13 secretion as antibody production matures suggesting early development of Th1 followed by Th2 and Treg $CD4^+$ T cells [32, 57, 80]. The possible development of effector $CD4^+$ T cells producing IL-17 during the response to MeV was suggested in a vaccine study, but has not been systematically evaluated [156].

Because it is likely that the functional evolution of T cell subsets during the prolonged phase of MeV RNA clearance is important for eventual virus clearance, immune suppression and establishment of life-long protective immunity, we characterized cellular immune responses to MeV over a period of six months after infection of rhesus macaques with a wild type strain of MeV.

4.3 MATERIALS AND METHODS

Animals, infection and sample processing

Five (14Y, 17Y, 31Y, 46Y and 50Y) 3-year-old male measles-naïve rhesus macaques (*Macaca mulatta*) were infected intratracheally with the Bilthoven strain of wild-type MeV, as previously described (See Chapter 2). Heparinized blood was collected from the femoral vein before infection and every 3-14 days after infection for six months. Beginning 10 days after infection, IDEXX Laboratories performed automated complete blood counts.

ELISPOT assays

Enzyme-linked immunosorbent spot (ELISPOT) assays were used to identify PBMCs producing IFNγ and IL-17. Multiscreen HTS HA Opaque 96-well filtration plates (Millipore) were coated with mouse anti-human IFNγ antibody (BD Biosciences, 2 μg/ml) or IL-17A antibody (eBioscience, 5 μg/ml) and blocked with RPMI/10% FBS. Cells were not stimulated or were stimulated with 1 μg/ml pooled hemagglutinin (H) or N overlapping peptides, 5.8 μg/ml MeV-infected Vero cell lysate (Advanced Biotechnologies) or 5 μg/ml concanavalin A (Con A). Freshly isolated PBMCs (10^5) were added to wells stimulated with Con A, H or N peptides and $5x10^5$ PBMCs were added to non-stimulated and MeV lysate wells and incubated at $37^oC/5\%CO_2$ for 40-42 hours. Biotinylated anti-human IFNγ (Mabtech, 7-B6-1; 1 μg/ml) or anti-human IL-17A (eBioscience, 64DEC17; 2 μg/ml) antibody was added for 2 hours. Plates were developed with avidin-horseradish peroxidase (BD Biosciences; 1:2000) and diaminobenzidine substrate. Plates were read and analyzed using an ImmunoSpot plate reader and ImmunoSpot 5.0 software (C.T.L.). Data are presented as spot-forming cells (SFCs)/10^6 PBMCs. MeV-specific SFCs were determined by subtracting the spontaneous (no *in vitro* stimulation) SFCs from the MeV-stimulated SFCs at each time point. All assays were done in duplicate.

Flow cytometry

Multicolor flow cytometry with intracellular cytokine staining was used to identify $CD4^+$ and $CD8^+$ T cells expressing IFNγ, IL-17, and RORγt. PBMCs were stimulated for 12 hours using pooled overlapping H or N peptides (1μg/ml), peptide diluent dimethyl

sulfoxide (DMSO) or staphylococcal enterotoxin B. Except where noted, all reagents were from BD Biosciences or eBioscience. Mouse anti-human CD28 (CD28.2) and anti-human CD49d (9F10) were included with the peptides and DMSO. All stimulation mixes included GolgiStop and GolgiPlug.

Live/Dead Fixable Violet Dead Cell Stain Kit (Invitrogen) was used to eliminate dead cells from the analysis. Prior to surface staining, cells were incubated with human FcR block. All panels included anti-CD3 antibody (SP34-2). For days 0-18 a "dump gate" was used to eliminate cells labeled with anti-human CD14 (M5E2), anti-human CD20 (2H7) or anti-human CD8 (SK1) and $CD4^+$ cells were defined as $CD14^-CD20^-CD3^+CD8^-$, because the anti-CD4 antibody initially used (RPA-T4) stained monkey $CD4^+$ T cells poorly. From day 28 onward, anti-human CD4 (Biolegend, OKT4) was used and $CD4^+$ T cells were defined as $CD14^-CD20^-CD3^+CD4^+$. $CD8^+$ cells were defined as $CD14^-CD20^-CD3^+CD8^+$.

Intracellular staining was done following fixation and permeabilization of cells. For days 7-21, the Cytofix/Cytoperm Fixation and Permeabilization Kit and subsequently the Foxp3 Staining Buffer Set was used. Intracellular staining was done to detect the transcription factor RORγt (Q21-559) and the cytokines IFNγ and IL-17A. T cell functionality was determined by gating on $CD3^+CD4^+$ and $CD3^+CD8^+$ cells stained for expression of IFNγ, tumor necrosis factor alpha (TNFα), IL-2 or CD107a. Boolean gating was used to define all possible subsets. Percentages of cells that expressed one, two, three or four different functional markers were grouped and relative frequencies for each subset

within $CD3^+CD4^+$ and $CD3^+CD8^+$ T cell populations were calculated. Samples were run on a BD FACS Canto II flow cytometer and analyzed using BD FACSDiva, FlowJo and SPICE (version 5.1; NIAID, NIH) software.

Statistics

The significance of differences in IFNγ and IL-17 secreting cells was assessed by a one-way ANOVA with repeated measures followed by Bonferroni's multiple comparisons test (GraphPad Prism version 7.00). Means for each time point were compared to day 0 pre-infection levels, a p-value less than 0.05 was considered significant.

4.4 RESULTS

Changes in circulating leukocytes

The numbers of total white blood cells, lymphocytes, and neutrophils in circulation were depressed during the viremia (day 10) but levels rebounded after the virus was cleared and then generally they were within the normal range by four weeks after infection (Figure 4-1a). Both $CD8^+$ and $CD4^+$ T cell numbers were decreased from day 10-18 and then returned to baseline (Figure 4-1b). There was a transient increase in the CD4:CD8 ratio as the viremia was cleared and then stabilized in the normal range (Figure 4-1c).

ELISPOT analysis of IFNγ- and IL-17 secreting cells

PBMCs secreting IFNγ (Figure 4-2a) and IL-17 (Figure 4-2d) as a result of *in vivo* activation were present at multiple times after infection with peaks at days 14-21, 52 and

126. *In vitro* MeV antigen stimulation resulted in a significant increase in MeV-responding IFNγ-secreting cells at day 21 after infection coincident with the clearance of infectious virus and decline in viral RNA levels (Chapter 2 Fig 2-2; Figure 4-2b). In contrast, peak production of IL-17 occurred later on days 52 and 126 as viral RNA was being cleared (Chapter 2 Fig 2-2; Figure 4-2e).

Both $CD4^+$ and $CD8^+$ T cells express IFN-γ during viral clearance and recovery

To determine the cellular sources and MeV protein-specific responses of early and late IFNγ production during MeV infection, PBMCs were stimulated with overlapping H or N peptides and analyzed by multi-parameter flow cytometry. $CD4^+$ and $CD8^+$ cells specific for both H and N proteins were sources of IFNγ throughout the course of infection (Figure 4-3a,b). $CD4^+$ T cells producing IFNγ in response to MeV peptide stimulation were most abundant in circulation at 10 and 84 days after infection (Figure 4-3a). Increased production of IFNγ by $CD8^+$ T cells was observed in all macaques at 10 days post-infection (Figure 4-3b). A second wave of IFNγ-producing $CD8^+$ T cells appeared from 84-140 days after infection in four of the five macaques (Figure 4-3b).

A series of studies have suggested that both the magnitude and quality of the T-cell response is important to controlling virus infection [157-160]. Determination of the quality of T cell responses includes assessing the diversity or polyfunctionality of these cells by measuring the simultaneous production of multiple cytokines [157, 161]. The polyfunctionality of H- and N-specific $CD4^+$ and $CD8^+$ T cells were assessed by determining the ability of cells to simultaneously express IFNγ, IL-2, TNF-α ($CD4^+$ and $CD8^+$) and CD107a ($CD8^+$ only). A large fraction of H-specifc CD4+ T cells were

polyfunctional (producing more than one cytokine) at 56 dpi (Figure 4-3c and 4-4a), while N-specific CD4+ T cells were more polyfunctional at 28 dpi (Figure 4-3c). In contrast, there was a large fraction of H- and N-specific polyfunctional CD8+ T cells at multiple time points after infection (Figure 4-3d and 4-5). Overall, $CD4^+$ T cells were polyfunctional primarily at earlier time points (Figure 4-3c and 4-4) while $CD8^+$ T cells were polyfunctional at both early and late phases of recovery (Figures 4-3d and 4-5). Thus, MeV infection induces prolonged multifunctional virus-specific T cell responses likely to be important not only for controlling and clearing infection but also for induction of long-term protective immunity.

MeV-specific $CD4^+$ and $CD8^+$ T cells begin producing IL-17 after clearance of infectious virus

To identify and further characterize the IL-17-producing cells induced during measles, surface and intracellular cytokine staining with multicolor flow cytometry was performed and a completed time-course analysis was completed on four of the five monkeys (Figure 3-6). All monkeys developed MeV-specific Th17 ($CD4^+IL\text{-}17^+$) cells (Figure 4-6a). H-specific Th17 cells increased from less than 0.01% (days 0 and 10) to 1.35-2.27% of the $CD4^+$ T cell population at day 18, followed by intermittent increases above baseline throughout the follow-up period (Figure 4-6a). N-specific Th17 cells were more variable. On average, Th17 H- and N- specific responses were highest on days 18 and 168 after infection.

MeV-specific Tc17 ($CD8^+IL\text{-}17^+$) cells were also induced after MeV infection with patterns of appearance in circulation similar to that of Th17 cells (Figure 4-6b). All

monkeys had an increase in H-specific Tc17 cells to 1.07-1.77% at day 18, whereas only monkey 31Y showed an increase in the frequency of N-specific Tc17 cells at day 18. Monkey 17Y had increases in both H- and N-specific Tc17 cells at day 56, while the other monkeys did not. All monkeys showed an increase in MeV-specific Tc17 cell frequencies at day 168 (Figure 4-6b). In summary, MeV H and N-specific $CD4^+$ and $CD8^+$ T cells producing IL-17 were detectable on day 18 when infectious virus was cleared and then again when viral RNA was no longer detectable in PBMCs.

MeV-specific T cell expression of RORγt

To better characterize the IL-17-producing cells, expression of the canonical IL-17 transcription factor RORγt [162] was examined (Figure 4-6a-d). The triphasic appearance of MeV-specific Th17 cells was also seen for cells expressing RORγt. All monkeys exhibited an increase in H-specific (1.09%±0.23) and N-specific (2.50%±1.99) RORγt-expressing $CD4^+$ cells at day 18 (Figure 4-6a). Frequencies of RORγt-expressing cells in response to H and N peptide stimulation remained low at day 28 through 39 followed by variable increases at day 56 or day 84. After day 113, the frequencies of MeV-specific RORγt-expressing $CD4^+$ T cells showed a steady increase to approximately 1-2% at day 168 (Figure 4-6a).

MeV-specific $CD8^+$ T cells also expressed RORγt (Figure 4-6b). H and N-specific increases were detected at 18, 56 or 64, and 168 days after infection. All monkeys displayed an increase in H-specific RORγt-expressing $CD8^+$ T cells at days 18 and 168 (Figure 4-6b). MeV-specific $CD8^+$ T cells expressing RORγt averaged approximately 4% of the total $CD8^+$ T cells, with individual frequencies ranging from 0.5-10%.

In addition, we also characterized the simultaneous expression of IL-17 and RORγt. To better visualize the dynamics of co-expression, histograms of $CD4^+IL\text{-}17^+$ cells and $CD4^+IL\text{-}17^-$ cells (Figure 4-6c) and $CD8^+IL\text{-}17^+$ and $CD8^+IL\text{-}17^-$ cells (Figure 4-6d) were created and overlaid. Prior to infection, all populations had very low levels of RORγt expression, but by day 18, RORγt expression was detected (Figures 4-6c,d). At days 56 and 113, IL-17-positive cells had higher levels of RORγt than IL-17-negative cells in both stimulated and non-stimulated populations. However, at day 168, the *ex vivo* MeV-stimulation does not increase RORγt expression in IL-17-positive relative to IL-17-negative cells.

4.6 DISCUSSION

Studies of measles in humans and macaques have suggested that T cell responses are important for recovery but the characteristics and time of appearance of MeV-specific T cells has received limited attention [49, 109]. In this study, we have monitored the evolution of T cell responses in MeV-infected rhesus macaques for a period of six months. Cells secreting IFNγ and IL-17 *ex vivo* and responsive to additional *in vitro* MeV stimulation appeared in circulation in multiple waves approximately 2-3, 8 and 18-24 weeks after infection without a change in total lymphocyte counts. IFNγ-secreting cells entered the circulation within the first 2 weeks and were most abundant 2-3 weeks after infection coincident with clearance of infectious virus. Virus-specific $CD4^+$ (Th1) and $CD8^+$ (Tc1) T cells were sources of IFNγ early in infection, while $CD8^+$ T cells were the predominant sources of IFN-γ later in infection. IL-17-secreting cells were most abundant later, were both $CD4^+$ (Th17) and $CD8^+$ (Tc17), expressed the transcription factor RORγt

and showed specificity for H and N MeV proteins. These data show an ongoing evolution of the virus-specific cellular immune response during MeV clearance and suggest that IFN-γ-production may be important for early viral control while IL-17 production may be more important late during clearance of viral RNA, maturation of the immune response and establishment of life-long protective immunity.

Many IFNγ -producing $CD4^+$ and $CD8^+$ T cells were polyfunctional, secreting IL-2 and TNF-α as well as IFN-γ. These data are consistent with previous observations of increases in levels of TNFα mRNA in PBMCs and of IFNγ, soluble IL-2 receptor, soluble CD8 and CD4 in plasma of children at the time of the measles rash [29, 55-57, 80]. IFN-γ has potent antiviral activities through induction of antiviral proteins and IFNγ- producing $CD8^+$ T cells are also likely to have cytotoxic activity, both of which contribute to clearance of many virus infections [163]. *In vivo* depletion of $CD8^+$ T cells has demonstrated their importance for control of measles virus [49], simian immunodeficiency virus (SIV) [164] and hepatitis B virus [165] infections. Furthermore, $CD4^+$ and $CD8^+$ T cells can develop into memory T cells, which are more efficient producers of IFNγ upon restimulation and provide protection from reinfection. The second increase in IFNγ-producing $CD8^+$ T cells in response to *ex vivo* stimulation 12-20 weeks after infection may reflect this population. Although more studies are needed to characterize these cells, it is likely that the T cell increase early after infection is important for the clearance of infectious virus while the later response may indicate renewed appearance of effector cells or development of a memory response.

A role for IL-17-producing cells in virus infection is less well characterized, but they have been implicated in impaired virus clearance and immunopathology as well as

improved outcome [166]. Th17 cells are induced from naïve $CD4^+$ T cells in the presence of IL-6 plus some combination of TGFβ, IL-21 and IL-1β; a process that is inhibited by type I IFN [167] and suppresses Foxp3 expression necessary for Treg development. The innate response that occurs after MeV infection may facilitate the development of IL-17-producing cells because IL-6 and IL-1β are increased early, but very little, if any, type I IFN is produced [29, 32, 35]. The time course for generation of IL-17-producing T cells has not been carefully assessed for most infections; however, a few studies suggest that peak production is often late. For instance, in mice with keratitis due to herpes simplex virus infection, two waves of IL-17 mRNA (day 2 and day 21) are observed [168]. In children with respiratory syncytial virus-induced bronchiolitis, levels of IL-17 in nasopharyngeal secretions are higher in convalescence than during acute disease, in contrast to the other cytokines and chemokines measured [169]. Previous studies of macaques have shown increases in IL-17-producing cells at 10 and 35 days after MeV infection, but later times were not assessed [156]. In this study we have shown that IL-17-secreting cells were not only produced early in infection (day 18), but also late during the recovery process (day 168) and that both $CD4^+$ and $CD8^+$ T cells are sources of IL-17. While Th17 cells are polyfunctional, have multiple phenotypes and can play both detrimental and beneficial roles in disease pathogenesis, its role in measles is still unknown [167, 170, 171].

Virus-specific Th17 cells have been detected as part of the $CD4^+$ T cell response to viral infections of mice [168, 172-176], nonhuman primates [177-179] and humans [180-182]. Expression of IL-17 by recombinant vaccinia virus leads to increased levels of virus in tissues [183]. Numbers of NKT cells producing IL-17 correlate with failure to

control chronic SIV infection in macaques and higher levels of circulating Th17 cells correlate with higher viral loads and more severe liver disease in humans with chronic hepatitis B virus infection [184]. However, both improved clearance of influenza virus from the lung [185] and no effect on clearance of herpes simplex virus from the cornea [186] are also reported in response to IL-17-producing cells. The mechanism by which IL-17-producing cells may inhibit virus clearance is not clear.

Many innate immune cells including innate lymphoid cells, NK cells, and NKT cells can produce IFNγ and/or IL-17. It is likely that these cells may contribute to the small burst of IFNγ and IL-17 detected at seven days post-infection by ELISPOT assay. However, by 10 dpi all macaques had developed rash, a hallmark for the onset of the adaptive immune response during MeV infection [22]. Therefore, adaptive immune cells are likely the predominant producers of IFNγ and IL-17 after the first week of infection.

These studies highlight the prolonged and complicated cellular immune responses generated by MeV infection. The immunologic processes driving the late development and repeated waves of IFNγ and IL-17-producing cells and the effects of these responses on MeV RNA clearance are not known and merit further investigation.

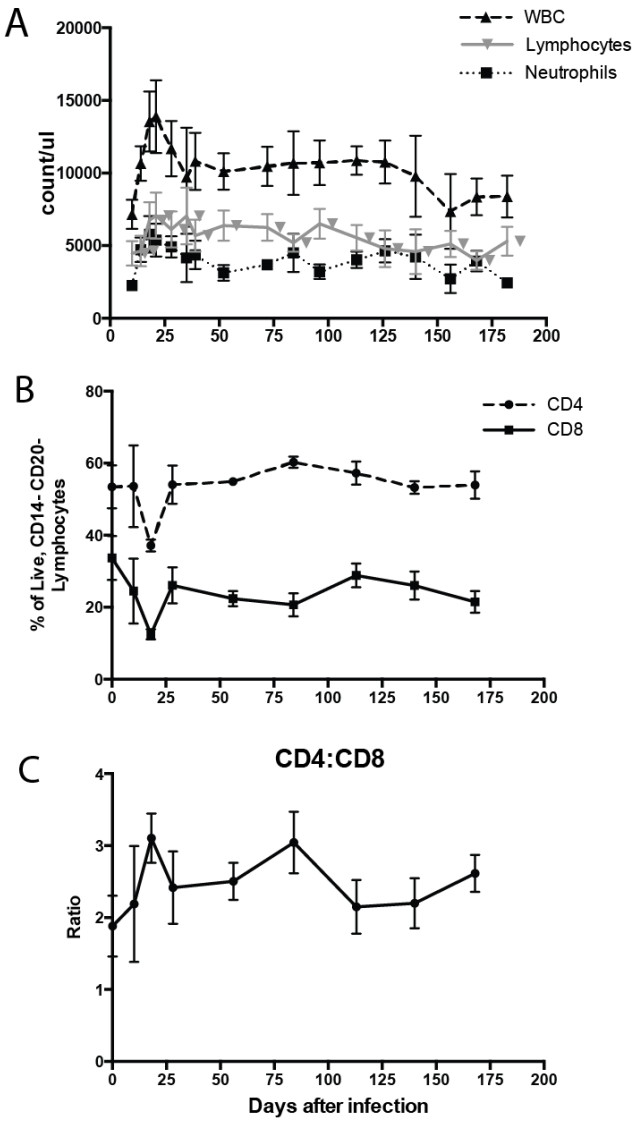


Figure 4-1. Changes in leukocyte counts after infection. Complete blood counts were used to measure the total numbers of circulating white blood cells (WBCs; dashed line), lymphocytes (gray line) and neutrophils (dotted line) (A). Normal ranges of cell counts for 3-4 year old rhesus macaques are: 7,700-13,000 WBCs/ml, 2,671-8,350 lymphocytes/ml, and 2,671-5,147 neutrophils/ml. Percentages of lymphocytes that were $CD4_+$ and $CD8_+$ T cells were determined by flow cytometry (B) and the CD4:CD8 ratio calculated (C). The normal CD4:CD8 ratio is 2.

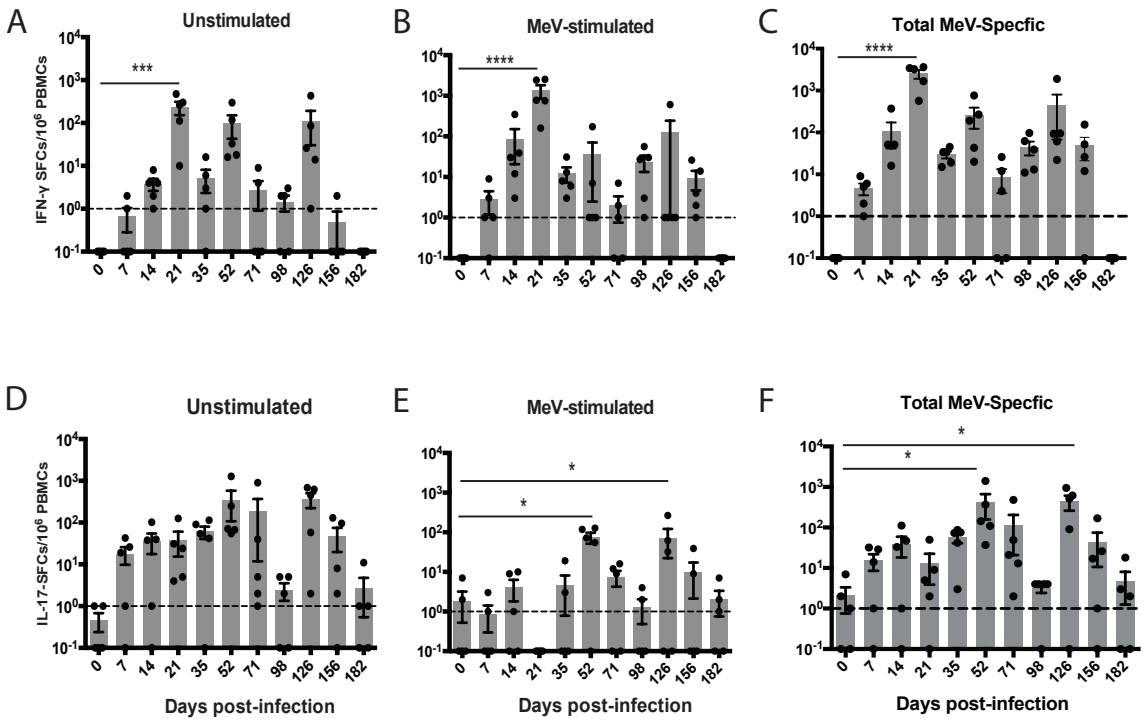


Figure 4-2. ELISPOT detection of IFN-γ and IL-17-secreting cells. PBMCs from MeV infected macaques were left unstimulated (A,D) or were stimulated with H and N peptides (B-C) or MeV lysate (E-F) and cultured on plates coated with antibody to IFN-γ (A-C) or IL-17A (D-F) to determine numbers of spot-forming cells (SFCs) per 10_6 PBMCs. To determine the numbers of cells producing IFN-γ (B) or IL-17 (E) in response to *in vitro* MeV stimulation, numbers of SFCs in unstimulated wells (A,D) were subtracted from the numbers of SFCs in MeV H and N or MeV lysate-stimulated wells. Total MeV-specific responses are also shown (C,F). Statistics used, one-way ANOVA with repeated measures followed by Bonferroni multiple comparisons test (n=5). *P<0.05; ***P<0.001; ****P<0.0001

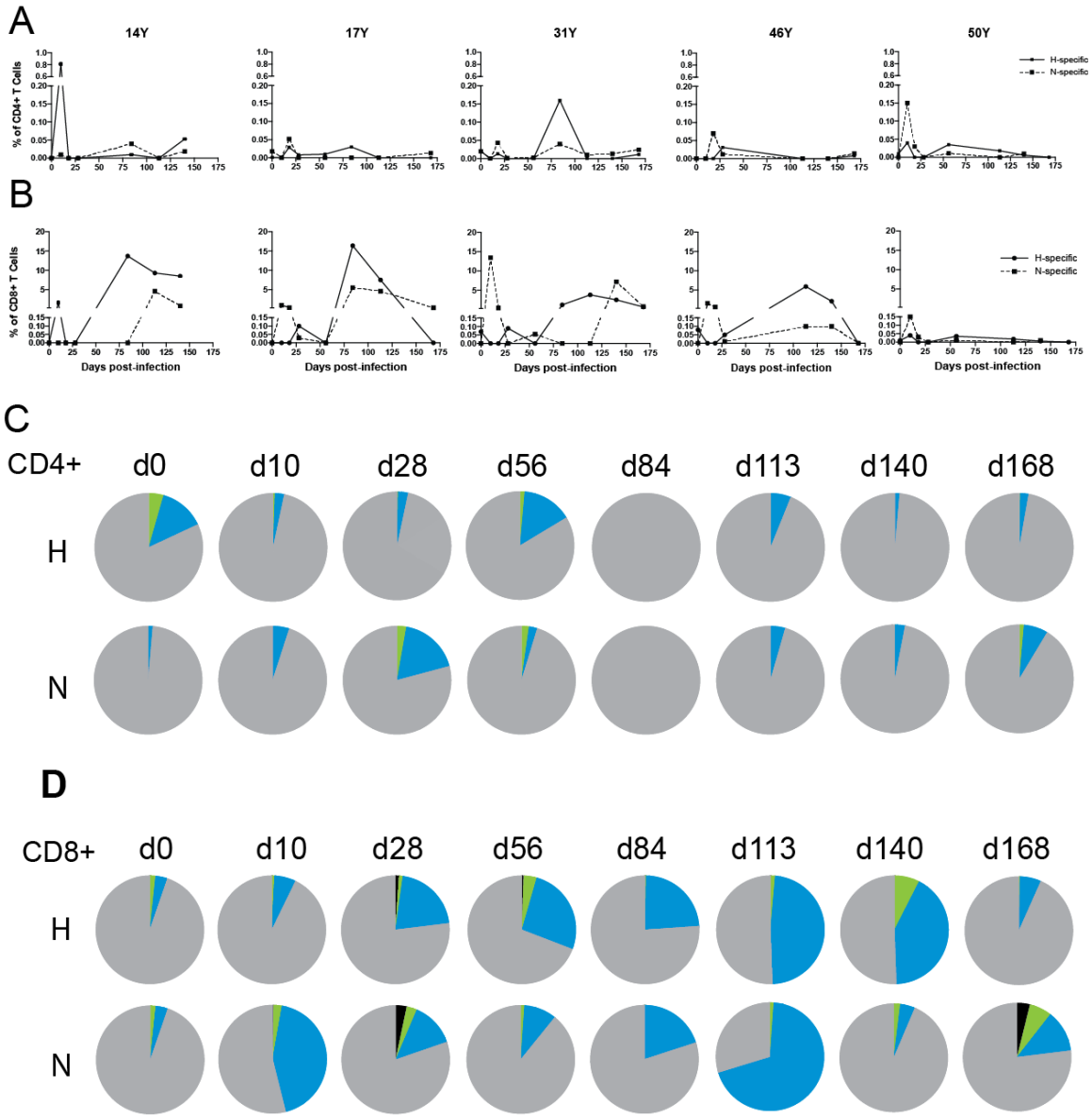

A
14Y
17Y
31Y
46Y
50Y
% of CD4+ T Cells
H-specific
N-specific
B
% of CD8+ T Cells
Days post-infection
C
CD4+
d0
d10
d28
d56
d84
d113
d140
d168
H
N
D
CD8+

Figure 4-3. Assessment of the ability of MeV-specific T cells to produce effector cytokines over time. PBMCs were stimulated with pooled overlapping peptides from the MeV H or N proteins and IFN-γ-producing CD4+ T cells (A) and CD8+ T cells (B) were identified. The frequency of MeV specific IFNγ-producing cells was determined by subtracting the spontaneous response. The polyfunctionality of the MeV-specific T cell response was assessed by determining the ability of CD3+CD4+ T cells to simultaneously express IFNγ, TNFα, or IL-2 (C) and CD3+ CD8+ T cells for their ability to simultaneously express IFNγ, CD107a, TNFα, or IL2 (D). Pie charts show the functional composition of $CD4_+$ and $CD8_+$ T cells that simultaneously express one (gray), two (blue), three (green), or four (black) different functional markers at a given time point (C, D).

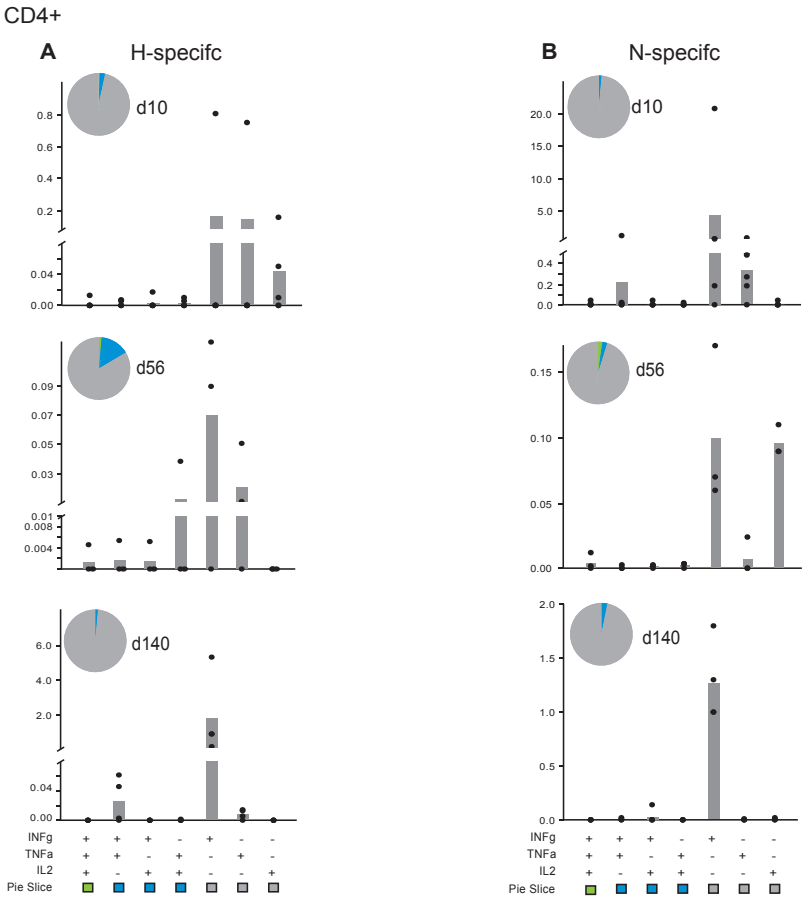


Figure 4-4. Functional analysis of MeV-specific CD4+ T cells. PBMCs were stimulated with pooled H- and N- peptides. CD3+CD4+ T cells were assessed for the ability to simultaneously express IFNγ, TNFα, or IL-2 (A and B). Subsets of cells expressing each functional marker were analyzed by Boolean gating. The frequency of each subset within CD4+ T cells is shown in the bar chart.

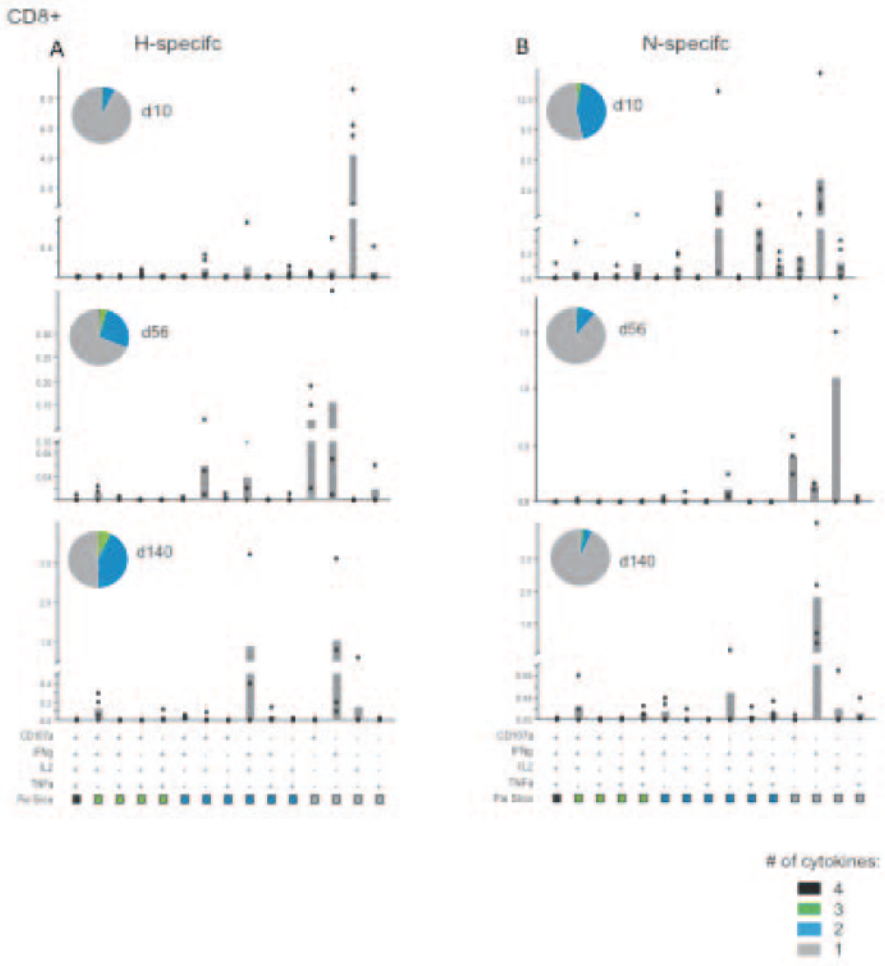


Figure 4-5. Functional analysis of MeV-specific CD8+ T cells. PBMCs were stimulated with pooled H- and N- peptides. CD3+CD8+ T cells were assessed for the ability to simultaneously express IFNγ, CD107a, TNFα, or IL-2 (A and B). Subsets of cells expressing each functional marker were analyzed by Boolean gating. The frequency of each subset within CD8+ T cells are shown in the bar chart.

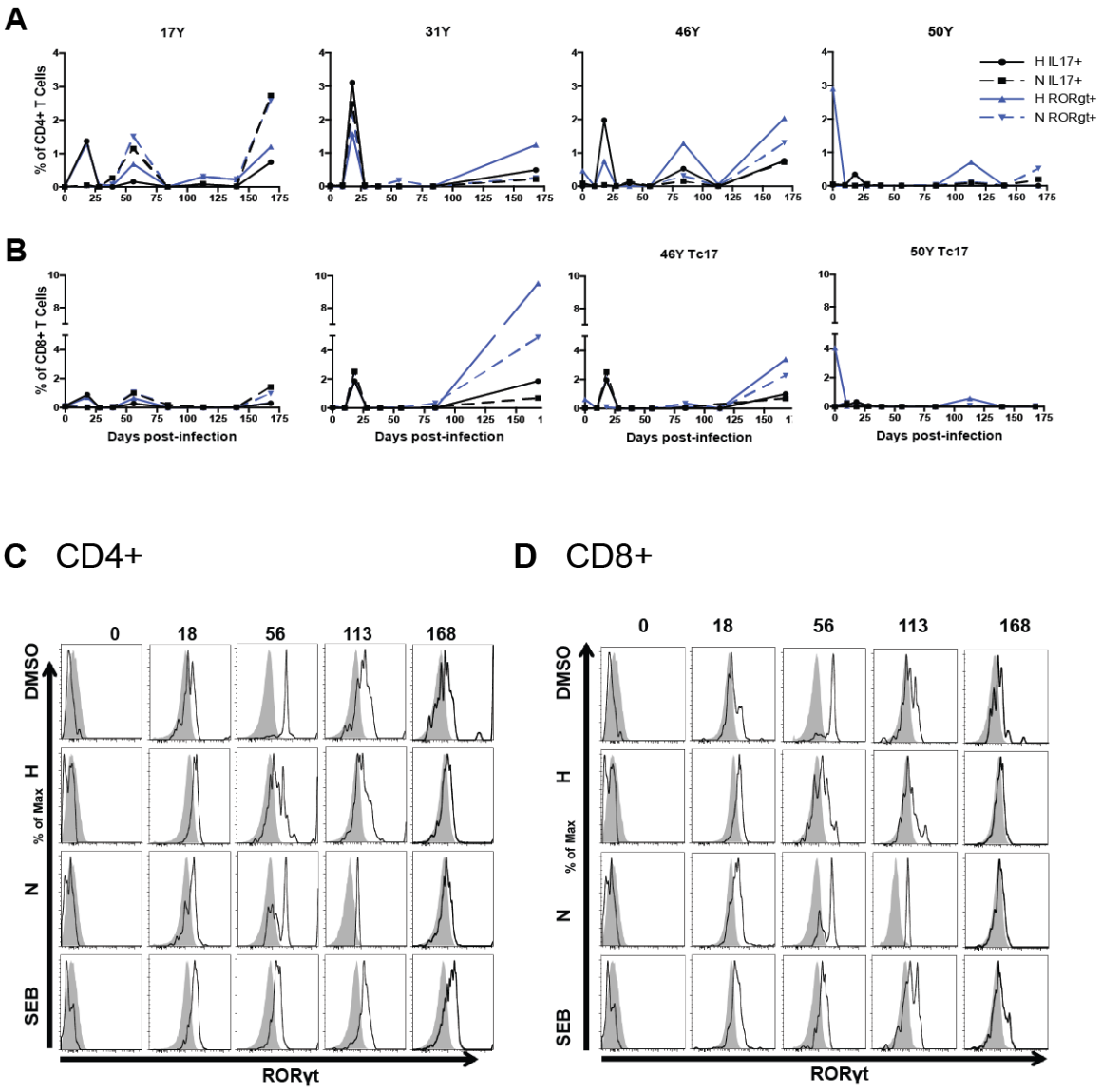


Figure 4-6. IL-17 and RORγt expression of MeV-specific T cells. PBMCs were stimulated with DMSO diluent, pooled overlapping peptides from the MeV H or N proteins or staphylococcal enterotoxin B (SEB) and IL-17 and RORγt-producing $CD4^+$ (A) and $CD8^+$ (B) T cells were identified by intracellular cytokine staining and multicolor flow cytometry. $CD4^+IL\text{-}17^+$ (open) and $CD4^+IL\text{-}17^-$ (gray) populations (C), and in $CD8^+IL\text{-}17^+$ (open) and $CD8^+IL\text{-}17^-$ (gray) populations (D) are overlaid for comparison of RORγt expression.

Chapter 5

Case Report: Measles-specific T cell responses promote virus clearance and recovery in the absence of antibody

5.1 ABSTRACT

Infection with wild-type measles virus (MeV) induces lifelong protection from reinfection. The humoral immune response is generally the primary focus for determining vaccine efficacy and levels of neutralizing antibodies correlate with protection against measles. However, a number of studies have demonstrated that the cellular immune response to measles is essential for virus clearance and recovery. We present a case in which failure to mount MeV-specific antibody responses after infection did not affect viral clearance or recovery. This case report confirms the importance of virus-specific T cell responses in facilitating measles virus clearance.

5.2 INTRODUCTION

Measles virus infection of immune-competent hosts elicits a robust virus-specific response that leads to virus clearance, recovery, and lifelong protection. The immune correlates of protection against measles have been defined, but the relative contributions of cellular and humoral immunity to virus clearance and recovery are not completely understood. Levels of MeV-specific neutralizing antibodies correlate with protection, however studies in non-human primates have suggested that humoral immunity plays a minimal role in infectious virus clearance [41, 49, 64].

Cellular immune responses are considered to be most important for clearance of MeV because children with B-cell deficiencies generally recover, while those with impaired cellular immunity often develop severe disease [154]. Moreover, measles in HIV-infected children leads to prolonged illness and MeV shedding and higher mortality, suggesting that impaired T cells responses results in slow viral clearance [107, 187]. This phenomena has also been observed in experimentally infected macaques, where depletion of $CD8^+$ T cells resulted in increased and prolonged viremia [49].

In this report, we describe an instance in which measles virus infection of a rhesus macaque failed to induce a robust virus-specific antibody response without apparent deficits in the cellular immune response. A lack of MeV-specific neutralizing antibodies after infection did not appear to have any clinical significance or impact on clearance of infectious virus and recovery. However, we did observe decreased production of MeV RNA in respiratory secretions and in the

blood. This suggests that MeV-specific T cell responses can prevent viral replication despite the absence of an antibody response.

5.3 CASE PRESENTATION

The case is a 3 year-old male rhesus macaque (24Y) infected intratracheally with the wild-type Bilthoven strain of measles virus as part of a vitamin A supplementation trial. In this study, monkeys received either two daily doses of vitamin A (100,000 units, Vitamin Angels, Santa Barbara, CA; 14Y, 24Y, 50Y) or placebo (17Y, 31Y, 46Y) upon rash development. Heparinized blood was collected before infection and every 3-14 days after infection for six months. Cellular and humoral responses were studied through six-months post-infection and it was observed that the monkey (24Y) did not develop MeV-specific neutralizing antibodies.

Virus Clearance

Infectious virus in the blood was monitored by co-cultivation of PBMCs with Vero/hSLAM cells. Similar to the rest of the cohort, 24Y developed a viremia by day 7 and cleared infectious virus from PBMCs by day 18 (Figure 5-1a). 24Y also developed a rash by 10 days post-infection (dpi) that was cleared by 14 dpi, however the rash was less extensive in comparison to the rest of the group (Figure 5-1a).

MeV RNA was measured in respiratory secretions and PBMCs by RT-PCR and qRT-PCR, respectively. In respiratory secretions, MeV RNA was only detectable at day 14 post-infection (Table 5-1). This was in contrast to the rest of the cohort that shed viral RNA beginning at 7-10 dpi through 2-3 weeks post-infection. Additionally,

24Y's MeV RNA levels in PBMCs were consistently below the limit of detection (Figure 5-1b).

Humoral immune response

Enzyme immunoassays were used to measure MeV-specific IgM and IgG in serum, as previously described (See Chapter 3). MeV-specific IgM in the blood of 24Y was detectable at low levels at 28 dpi, one week after the rest of the cohort (Figure 5-2a). IgG levels, when detectable, were consistently lower in quantity and avidity (Figure 5-2b,d). By day 84 post-infection, MeV-specific IgG was no longer detectable. Neutralizing antibody concentration in serum was measured using plaque reduction neutralization assay and was not detected at any point after infection (Figure 5-2c). To define the specificity of these antibodies, we used enzyme immunoassays specific to measles hemaglutinin (H), nucleocapsid (N), and fusion (F) proteins. The antibody produced was predominantly F-specific and detectable for 2-3 weeks after infection (Figure 5-3c). 24Y did not develop robust antibody responses specific for –H and –N antigens (Figure 5-3a,b). Total IgG levels in serum were measured using an enzyme immunoassay to confirm that 24Y did not have any general defects in antibody development. Total IgG levels for 24Y were comparable to the rest of the monkeys (Figure 5-4).

B cell ELIspot assays were used to measure the production of virus-specific antibody secreting cells (ASCs) in the blood and bone marrow after infection. Antibody secreting cells were not detectable in the blood from 24Y until approximately two months post-infection, increased through three months post-

infection, and steadily declined to undetectable levels by six months post-infection (Figure 5-5a). In the bone marrow, there was prolonged, low level presence of antibody secreting cells through three months post-infection (Figure 5-5b). This is in contrast to the rest of the cohort who exhibited prolonged presence of antibody secreting cells through six months post-infection.

Histology

Inguinal lymph node biopsies were collected 71 and 154 days post-infection. Tissues sections were stained with hematoxylin and eosin. There were no distinct changes in tissue pathology in comparison to the rest of the group (Figure 5-6). We observed an increase in the number of germinal centers over time; however, MeV RNA was not detectable in the lymph node at 71 or 154 days post-infection.

Cellular immune response

ELISpot analysis was performed on PBMCs, as previously described (See Chapter 4), to measure MeV antigen-specific IFNγ (Figure 5-7a) and IL-17 (Figure 5-7b) producing cells. IFNγ-producing cells were present at multiple times after infection with peaks at 21, 72, and 155 dpi. Peak production of IL-17-producing cells occurred at 52 and 126 dpi. Overall, no significant defects in MeV-specific cellular immunity of were detected although his Th17 response was not as robust as the rest of the cohort.

5.4 DISCUSSION

We have presented a case in which virus-specific neutralizing antibodies were not produced in a rhesus macaque after wild-type measles infection. Despite this defect in humoral immunity, there was a robust antigen-specific T cell response. Upon infection, viremia was established, therefore, any deficiencies observed in viral immunity were not due to lack of a productive infection. Moreover, 24Y did develop the prototypical maculopapular rash associated with measles virus infection. In measles, the rash is a manifestation of the virus-specific cellular immune response. HIV-infected individuals can develop measles without exhibiting the characteristic rash [125, 188]. These data further confirm our observation that a robust MeV-specific cellular immune response developed despite the absence of antibody.

Measles is an acute, systemic viral infection and we have previously shown that the clearance of viral RNA from the blood and other tissues is prolonged and much slower than clearance of infectious virus [23]. In a previous study, rhesus macaques that received a live attenuated measles vaccine through a nebulizer, developed a T cell response but no neutralizing antibody. Upon challenge, these T cell primed macaques were not protected from viremia or rash, but cleared viral RNA more rapidly than unimmunized macaques [109]. In the case presented here, MeV RNA was cleared rapidly from nasal secretions and was consistently below the limit of detection in PBMCs. These data support the notion that T cell immunity may not be important in protection from acute disease but facilitates virus clearance and recovery.

Upon encountering antigen, activated B cells differentiate into either short-lived antibody-secreting cells, or undergo somatic hypermutation and affinity maturation in germinal centers and differentiate into memory B cells and long-lived plasma cells. Normally, MeV infection induces a robust virus-specific humoral response characterized by prolonged production of high affinity antibodies and antibody secreting cells. In the present case, MeV-specific IgG was detected intermittently for a short period of time suggesting the generation of short-lived antibody secreting cells. In addition, antibody-secreting cells were only detectable for a short period of time in the blood and bone marrow. The majority of neutralizing antibodies to MeV are specific for the H-protein, however F-specific antibodies can also be neutralizing. In the present case, the short-lived antibodies that were produced were specific for F and displayed no neutralizing activity.

Natural MeV infection generates long-lasting immunity that includes both MeV-specific antibody and T cell responses. While high titer, high avidity neutralizing antibodies protect against reinfection from measles, the independent roles of antibody and T cell responses in disease is not fully understood. In general, cellular immune responses to measles have been regarded as most important for virus clearance and recovery. We presented a case in which failure in the development of a robust MeV-specific antibody response had no impact on disease progression, but did prevent MeV replication in multiple tissues during recovery. This case highlights the importance of cellular immunity to measles and its independent role in the disease.

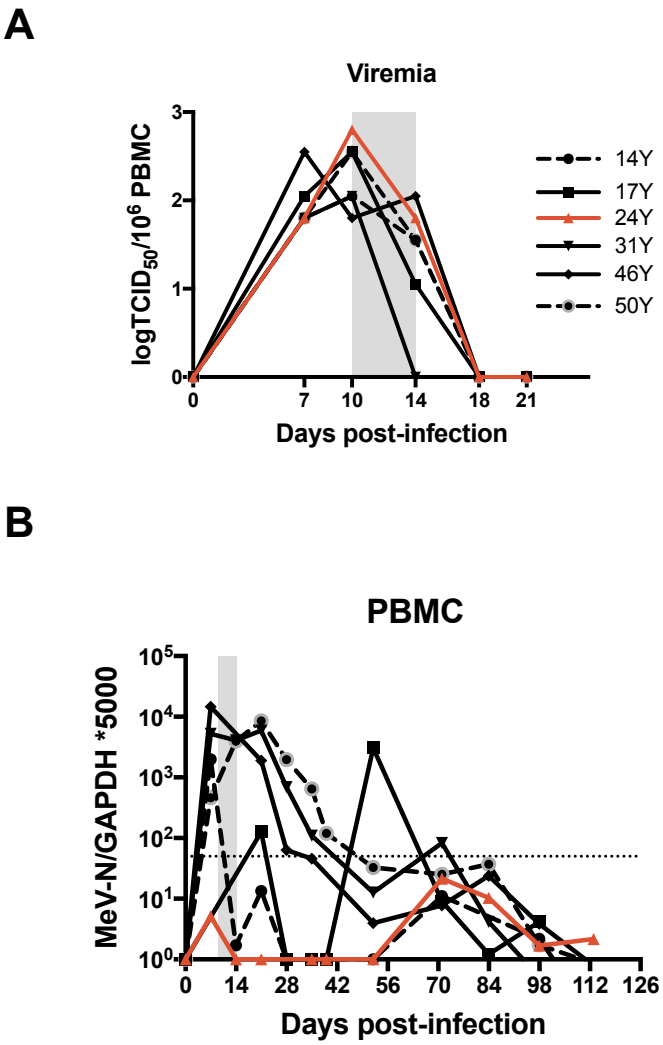
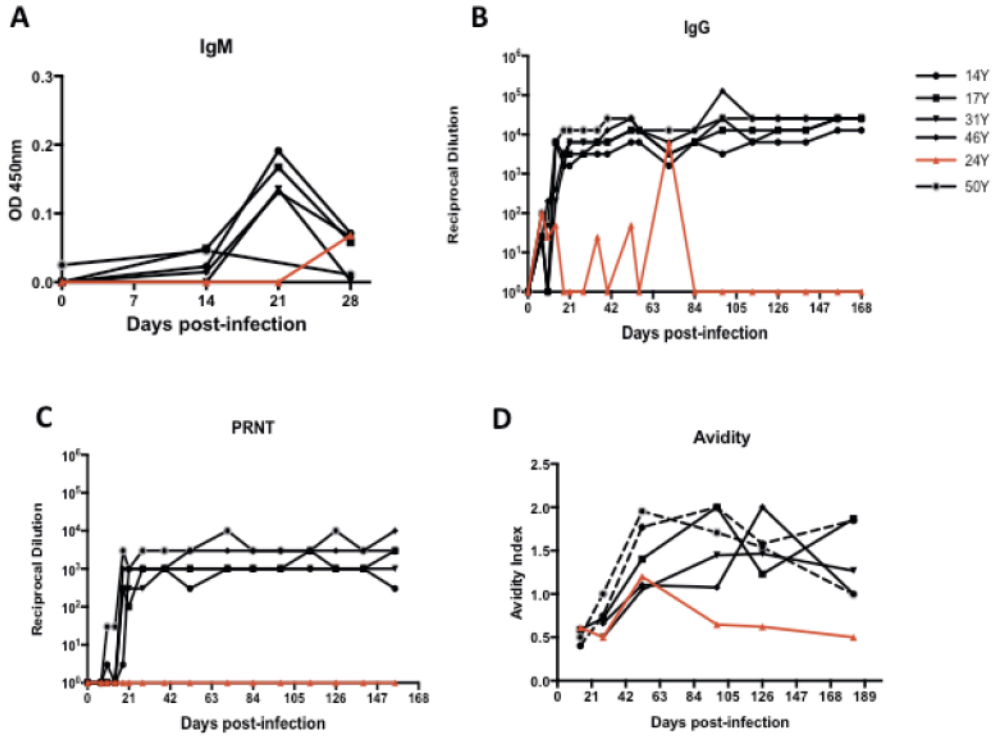


Figure 5-1. Virus clearance. Viremia was measured by co-cultivation of serially diluted PBMCs on Vero/hSLAM cells (A). Data are displayed as the log($TCID_{50}$)/10^6 PBMCs. Shaded area indicates period of rash. MeV N gene RNA in PBMCs was measured by qRT-PCR (B). Assays were run in duplicate with a standard of 10^1-10^8 copies of MeV RNA, the dotted line shows the limit of detection of the standards. MeV RNA load was normalized to the GAPDH control. Results are expressed as [(MeV N RNA copies)/(GAPDH RNA copies)]*5000.

	14Y	17Y	24Y	31Y	46Y	50Y
0	-	-	-	-	-	-
7	-	+	-	-	-	+
10	+	+	-	+	+	+
14	+	+	+	+	+	+
18	-	+	-	+	-	+
21	-	+	-	-	-	+
39	-	-	-	-	-	-

Table 5-1. Detection of MeV RNA in nasal secretions. To monitor MeV shedding from the respiratory tract, nasal secretions were collected from both nostrils with sterile cotton swabs and immersed in PBS. Cells and fluid were separated via centrifugations. RNA was extracted from cell pellets. MeV N gene RNA was measured by RT-PCR and results were read as positive (+) or negative (-)


A
IgM
OD 450nm
Days post-infection
B
IgG
Reciprocal Dilution
Days post-infection
14Y
17Y
31Y
46Y
24Y
50Y
C
PRNT
Reciprocal Dilution
Days post-infection
D
Avidity
Avidity Index
Days post-infection

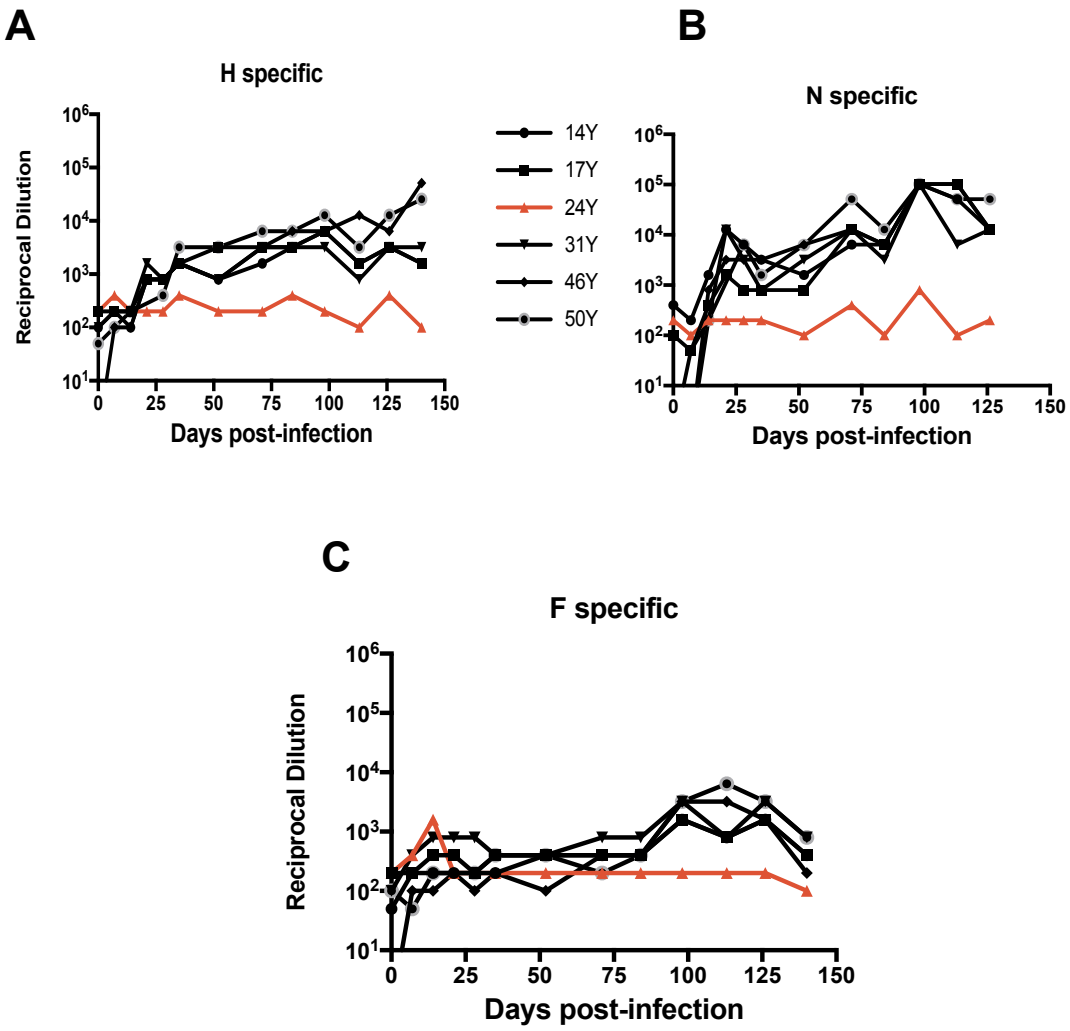


Figure 5-3. Antibodies to various MeV proteins. EIAs were used to measure antibodies specific for the MeV -H (A), -N (B), or –F (C) antigens in plasma. The EIA titer was the highest dilution at which responses were twice the background.

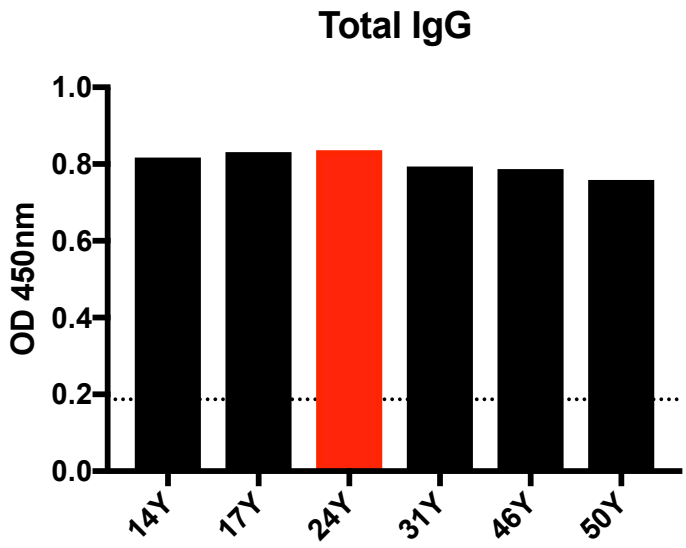


Figure 5-4. Total serum IgG levels. Serum was diluted 1:400 and total IgG was measured using an enzyme immunoassay. Plates were read at 450nm. The dotted line indicates an O.D. value of twice the background.

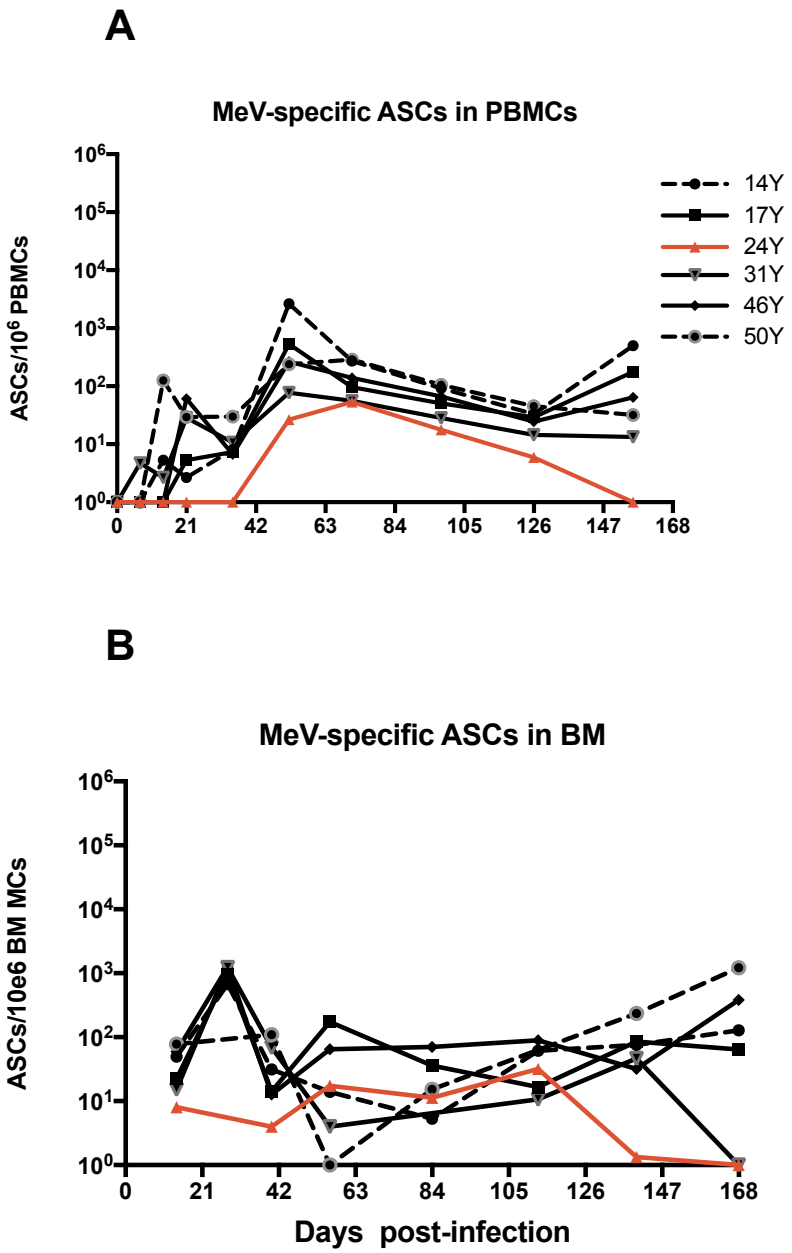
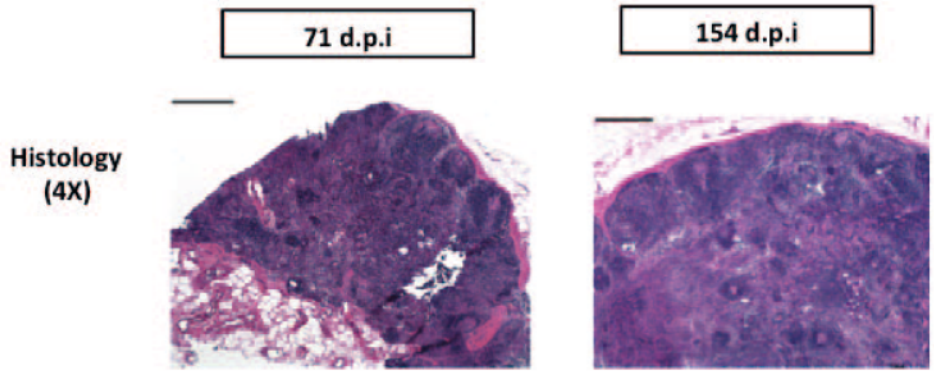


Figure 5-5. Production of antibody-secreting cells during the course of infection. The production of MeV-specific antibody-secreting cells (ASCs) in plasma (A) or bone marrow (B) was characterized using a B-cell ELISpot assay. Plates were coated with measles virus lysate and $5x10^6$ cells were added to each well. All samples were run in duplicate and data are plotted as mean ASCs per 10^6 PBMCs.


71 d.p.i
154 d.p.i
Histology
(4X)

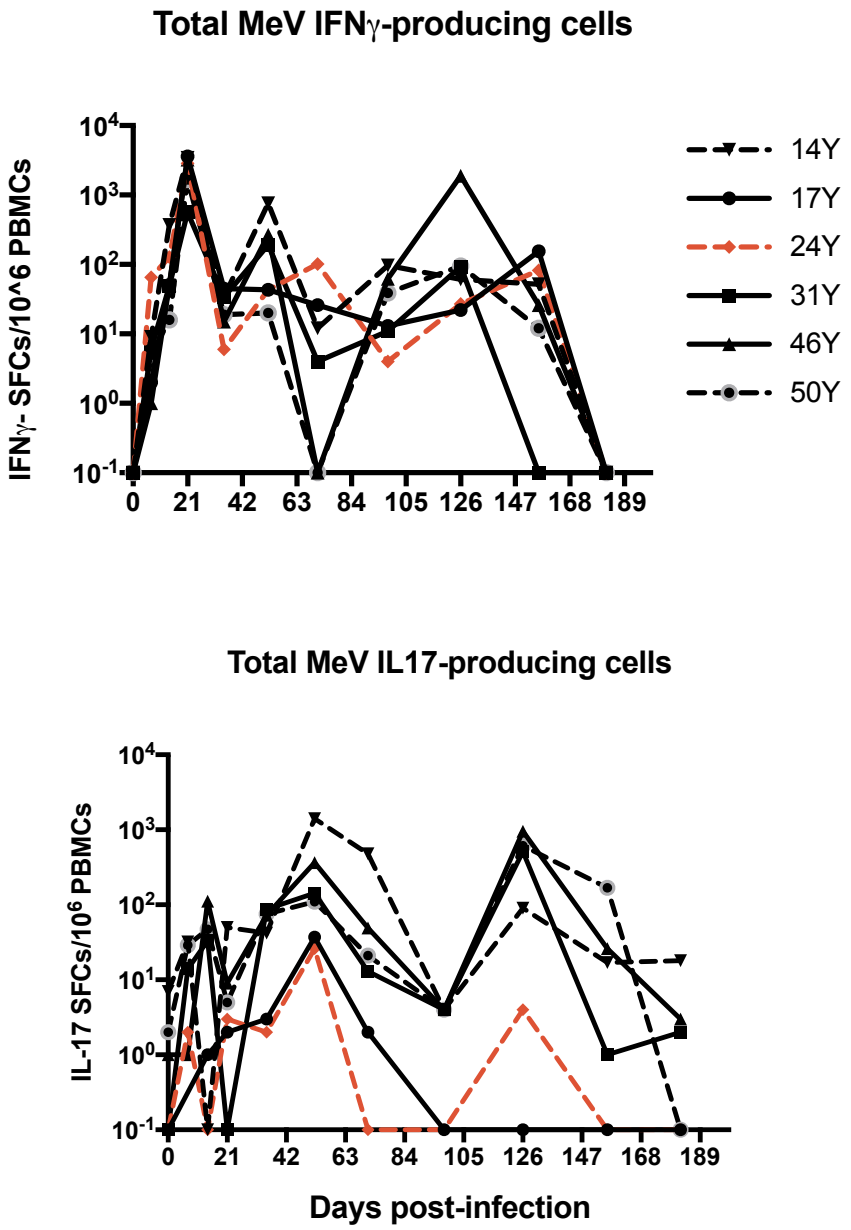


Figure 5-7. ELISPOT detection of IFN-γ and IL-17-secreting cells. PBMCs from MeV infected macaques were stimulated with H and N peptides (A) or MeV lysate (B) and cultured on plates coated with antibody to IFN-γ (A) or IL-17A (B) to determine numbers of spot-forming cells (SFCs) per 10^6 PBMCs.

Chapter 6

Measles virus-specific T cell responses in young adults 5 years after a third dose of MMR vaccine

6.1 ABSTRACT

Measles is a highly contagious systemic viral infection. Two doses of measles, mumps, and rubella (MMR) vaccine are highly effective, however, secondary vaccine failures occur about 5% of the time. We assessed measles-specific T cell responses in 31 young adults five years after receipt of a third dose of MMR (MMR3). Overall, there was a very minimal boost in MeV antigen-specific IFNγ or IL-17-producing T cells. As most subjects were seropositive prior to MMR3 receipt, a routine third dose of MMR does not appear to significantly boost measles immunity.

6.2 INTRODUCTION

Measles is a highly contagious, vaccine preventable disease. Despite the availability of a safe and effective vaccine, measles remains a major cause of morbidity and mortality globally among young children. Measles is typically a self-limiting disease characterized by fever, coryza, conjunctivitis, and rash. Recovery from infection results in life-long immunity. A number of live-attenuated measles vaccines are available (Chapter 1, Figure 1-5). In the U.S., the measles vaccine is administered to children as multivalent measles, mumps and rubella (MMR) vaccine.

The current vaccination schedule is designed to provide optimal efficacy by administering the measles vaccine to infants following the decay of maternal antibody, but before the likelihood of exposure [93]. A live-attenuated vaccine against measles is administered to children in two doses. The WHO recommends administration of the first dose at nine months in measles endemic regions and 12-15 months in non-endemic regions. About 85% of children develop protective immunity to measles when vaccinated at 9 months, while 95% will develop protective immunity when vaccinated at 12 months [22]. Because measles is highly infectious with a reproductive number of 12-15, administering a second dose of the vaccine ensures that population immunity will reach sufficiently high levels (92-95%) to interrupt transmission.

Two doses of the MMR vaccine are typically highly effective and sufficient to provide long-lasting immunity, however measles outbreaks have been reported among immunized individuals [139]. In addition, while most vaccinated individuals remain measles seropositive, antibody titers decrease 8-15 years post-vaccination [189, 190]. Waning immunity, and possibly secondary vaccine failures, can result in a shift in the age

distribution of measles from young children (< 5 years) to adolescents and young adults. In this study done in collaboration with the Centers for Disease Control (CDC), a third dose of MMR (MMR3) vaccine was administered to young adults more than 10 years after the second dose. Antibody and T cell responses were assessed in a previous study at 1 month and 1 year post-MMR3 vaccination. In this report, IFNγ and IL-17 ELISpots were used to assess MeV-specific T cell responses five years post-MMR3 vaccination.

6.3 MATERIALS AND METHODS

Study Subjects

Participants in this study were young adults age 18-24 years old who had previously received the MMR vaccine at Marshfield Clinic in Marshfield, Wisconsin. These subjects were part of the "MMR2 study" that examined immune responses in 612 children after administration of a second dose of MMR vaccine. In the MMR2 study, cellular immune responses were assessed in a subgroup of 60 children. As part of the current "MMR3 study", subjects from the MMR2 study (n=35) along with subjects not part of this study but of a similar age (n=15) received a third dose of MMR vaccine. Previous studies assessed their immune responses both at 1 month and 1 year after receipt of their third dose of MMR [191]. For this study, we have measured the cellular responses of 31 subjects five years after their 3rd dose of MMR.

Cells

Cryo-preserved PBMCs were thawed and washed twice with RPMI media supplemented with 10% human serum type AB. Cells were then cultured overnight at 37°C, 5% CO_2 in

RPMI media supplemented with 4% human serum type AB, 1% penicillin/streptomycin, and 1% 200 mM L-glutamine. Cultured PBMCs were then used for IFNγ and IL-17 ELISpot assays.

ELISPOT assays

Enzyme-linked immunosorbent spot (ELISPOT) assays were used to identify IFNγ and IL-17 producing cells. Multiscreen HTS HA Opaque 96-well filtration plates (Millipore) were coated with mouse anti-human IFNγ antibody (BD Biosciences, 2 μg/ml) or IL-17A antibody (eBioscience, 5 μg/ml) and blocked with RPMI medium (RPMI + 4% human serum AB + 1% penicillin/streptomycin, and 1% 200 mM L-glutamine 1%). Cells were not stimulated or were stimulated with 1 μg/ml pooled hemagglutinin (H) or (N) overlapping peptides, or 5 μg/ml concanavalin A. PBMCs (10^5) were added to wells stimulated with ConA, and $5x10^5$ PBMCs were added to H- or N-stimulated and unstimulated wells. Cells were incubated at $37^oC/5\%CO_2$ for 40-42 hours. Biotinylated anti-human IFNγ antibody (Mabtech, 7-B6-1; 1 μg/ml) or anti-human IL-17A antibody (eBioscience, 64DEC17; 2 μg/ml) was added for 2 hours. Plates were developed with avidin-horseradish peroxidase (BD Biosciences; 1:5000) and diaminobenzidine substrate (Invitrogen). Plates were read and analyzed using an ImmunoSpot plate reader and ImmunoSpot 5.0 software (C.T.L.). Data are presented as spot-forming cells (SFCs)/10^6 PBMCs. MeV-specific SFCs were determined by subtracting the spontaneous (no *in vitro* stimulation) SFCs from the MeV-stimulated SFCs. All assays were done in duplicate.

6.4 RESULTS

Measles virus-specific T-cell responses of 31 subjects 5 years after MMR3 receipt were assessed by IFNγ ELISpot (Fig 6-1a). ConA stimulation of PBMCs from all subjects were positive, ensuring that a lack of response was not due to the inability of cells to be stimulated. Virus-specific responses were determined by stimulating cells with pooled overlapping peptides specific for the measles hemagglutinin (H) or nucleocapsid (N) protein. The mean number of IFNγ spot forming cells (SFCs) per million PBMCs were 110.2± 391.7 with H-specific and 8.2± 12.9 with N-specific peptide stimulation (Figure 6-1a;Table 6-1). Due to large outliers, median values were also determined. The median SFC was 0 for the non-specific (unstimulated) response. The medians for peptide stimulation responses were 2 SFCs for H-specific and 4 SFCs for N-specific responses (Table 6-1). In general, most subjects had a much higher H-specific versus N-specific response while 10 of 31 (32.3%) subjects had no detectable virus-specific response (Figure 6-1). The majority of responding subjects had H- and N-specific responses above the threshold, based on background responses, of 20 SFCs per million PBMCs (Figure 6-2; Table 6-2)

PBMCs were available from a subset of 16 subjects to assess IL-17 producing cells 5 years post-MMR3 vaccination. Most subjects exhibited no IL-17 related cellular immune responses (Figure 6-1b). Two of the 16 subjects had an H-specific response and two of 14 subjects responded to N peptide stimulation. Mean MeV-specific responses were 0.95 ± 2.47 for H-specific responses and 0.50 ± 0.69 SFCs per million PBMCs for N-specific (Table 6-1). All responses were below the threshold of 20 SFCs per million

PBMCs (Table 6-2). Four of the 16 subjects did not respond to ConA stimulation (Figure 6-1b).

6.5 DISCUSSION

Cell-mediated immunity (CMI) of 31 subjects five years after receipt of a third dose of MMR was assessed using IFNγ ELISpot assays. Among the subjects tested, 21 (67.7%) exhibited MeV antigen-specific IFNγ production by T cells. These data suggest memory T cell responses have been maintained and can be detected five years post-vaccination in most subjects. Memory T cell responses have been reported to increase initially after measles vaccination and can still be detected seven years post-vaccination in children and adults [192]. Previous studies involving a subset of subjects from this MMR3 cohort assessed CMI responses at baseline (pre-vaccination), at 1-month, and 1-year post-vaccination. These studies found that while T cell responses were detected after a third dose of vaccination, they were not significantly increased when compared to baseline levels [191]. In a previous study of macaques vaccinated with a dry powder formulation of live-attenuated measles virus, only small increases in T cell responses were reported after challenge [193].

The role of Th17 cells in primary immune responses against many infections have been well documented, however recent advances suggest that Th17 cells may play a critical role in vaccine-induced immunity. There is growing evidence that long-lived, robust memory Th17 cell populations exist, exhibit some polyfunctionality, and are induced during vaccination to various pathogens [194]. IL-17 appears to be a major modulator in the generation of protective immunity in response to whole cell pertussis

[195] and rotavirus (VP-6) vaccination [196]. The role of Th17 cells in natural or vaccine-induced measles immunity is still unknown. However, we have shown that MeV-specific Th17 cells could not be detected in most subjects 5 years post-MMR3 vaccination. It is possible that Th17 cells may be transiently induced immediately after vaccination and are not as long-lived as IFNγ-producing T cells. This suggests that Th17 cells may play a minimal role in protective immunity to measles.

In summary, we were able to detect measles antigen-specific IFNγ producing T cells in the majority of subjects five years after receipt of the third dose of MMR. In contrast, MeV-specific IL-17 producing T cell responses were all below a threshold of 20 S.F.C. per 10^6 PBMCs. Therefore, IFNγ-producing memory T cells may play a role in protective immunity to measles while Th17 memory cells may not. Additionally, while measles-specific cell mediated responses can be detected years after receipt of a third dose of MMR, further studies are necessary to support the need for administration of a third dose to vaccinated populations.

Stimulation:	Unstimulated	H-specific	N-specific
IFNγ			
Mean S.F.C. ±SD (n)	4.11 ± 8.00 (31)	110.20 ± 391.70 (31)	8.34 ± 12.86 (30)
Median S.F.C.	0	2.00	4.00
IL-17			
Mean S.F.C. ±SD (n)	0 (16)	0.95 ± 2.47 (16)	0.50 ± 0.69 (14)
Median S.F.C.	0	0.1	0.1

Table 6-1. Summary of measles-specific IFN-γ and IL-17 producing cells 5-years post third dose of MMR. PBMCs were isolated and stimulated with either media alone (unstimulated), pooled H peptides, or N peptides. The table shows mean and median IFNγ and IL-17 spot forming cells (S.F.C.) per 10^6 PBMCs.

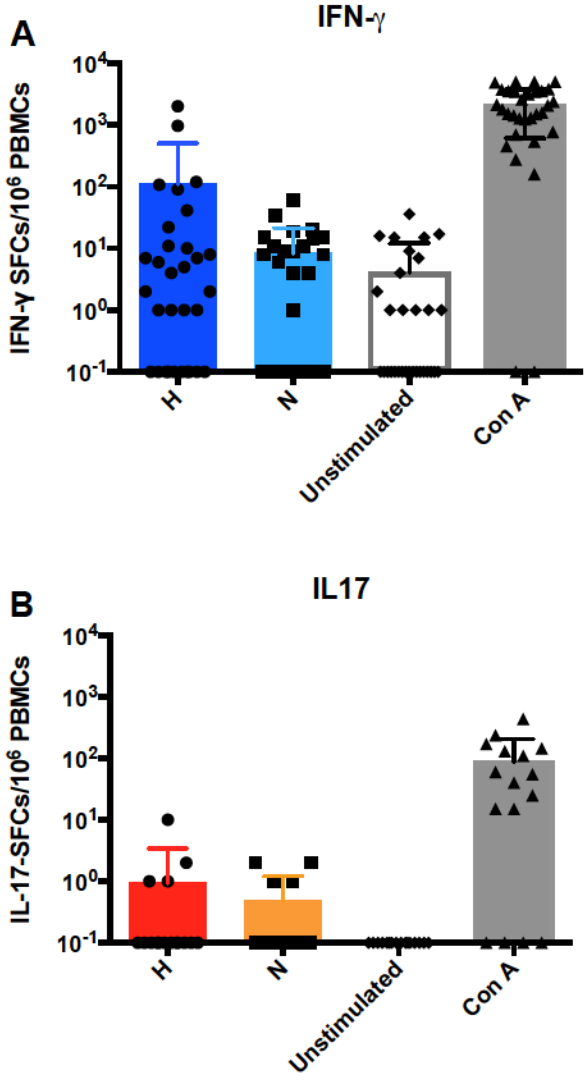


Figure 6-1. Detection of IFN-γ and IL-17-secreting cells. PBMCs from subjects were left unstimulated or were stimulated with H and N peptides or Concanavalin A and cultured on plates coated with antibody to IFNγ (A) or IL-17A (B) to determine numbers of spot-forming cells (SFCs) per 10^6 PBMCs. To determine the numbers of cells producing IFNγ (A) or IL-17 (B) in response to *in vitro* MeV stimulation, numbers of SFCs in unstimulated wells were subtracted from the numbers of SFCs in MeV H and N stimulated wells.

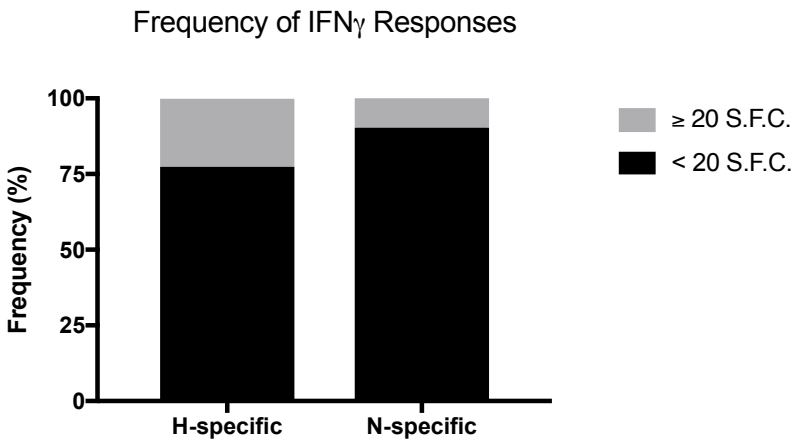


Figure 6-2. Frequency of MeV-specific T cell responses among subjects. The histogram shows the percentage of subjects that had H- or N-specific IFNγ S.F.C. per 10^6 PBMCs less than 20 (black bars) or greater than and equal to 20 (grey bars).

Stimulation:	H-specific	N-specific
IFN-γ		
< 20 S.F.C.	24 (77.4%)	28 (90.3%)
≥ 20 S.F.C.	7 (22.5%)	3 (9.7%)
IL-17		
< 20 S.F.C.	16 (100%)	14 (100%)
≥ 20 S.F.C.	0 (0%)	0 (0%)

Table 6-2. Summary of the frequency of MeV-specific IFNγ and IL17 responses among subjects. The table shows the number and percentage of subjects that had H- or N-specific IFNγ or IL-17 S.F.C. per 10^6 PBMCs less than 20 or greater than and equal to 20.

Chapter 7

General Discussion and Conclusion

The immune response to measles virus infection is a complex cascade of innate, cellular, and humoral immune responses leading to eventual recovery and the establishment of lifelong immunity. Although measles is a well-known disease that has been extensively studied, many aspects of disease pathogenesis remain unclear. Experimental infections of non-human primates have helped to improve our overall understanding of the pathogenesis of measles, and have been especially vital in studying early events before the onset of rash and late events after apparent recovery.

We have utilized a rhesus macaque model to assess viral RNA clearance, innate immunity and the evolution of the adaptive immune response during a primary wild-type measles virus infection. Collectively, this work was able to model the dynamics of various aspects of the host immune response to measles over an extended period of time, and develop our understanding on the role of MeV RNA persistence during infection. We found that viral RNA was cleared from the blood by two to three months post-infection, but still detectable in lymphoid tissue through five months post-infection. In addition, we demonstrated that viral RNA preferentially persisted in B cells in the periphery and in both macrophages and B cells in lymphoid tissue. This work offers new insights into various aspect of measles pathogenesis including prolonged immune suppression, long-lived immunity, and late development of progressive neurologic disease. Next, we evaluated the ability of MeV to regulate type I IFN responses. The data obtained here

suggest that while type I IFN is not induced during the innate response to measles, other innate responses are likely to play a role in activating the adaptive immune response.

Moving on to characterization of the dynamics of the adaptive immune response, our evaluation of the humoral response has shown that WT measles virus infection results in the prolonged induction and maturation of antibody responses. This work has aided in our understanding of the mechanism behind the development of long-term immunity associated with measles. We analyzed the appearance and function of MeV-specific T cells over six months. These studies showed the prolonged and ongoing evolution of the cellular immune response generated by MeV infection, and suggest that polyfunctional T cells are important for viral clearance and recovery. Lastly, we evaluated cellular immunity in a cohort of young adults after receipt of a third dose of MMR. This study was done in collaboration with the CDC and these results from this data will aid in our understanding of measles vaccine long-term immunity.

MeV RNA persistence: Mechanism and Consequences

Our work demonstrates that MeV RNA persists in several locations for many weeks to months after the clearance of infectious virus. Measles-associated immune suppression has long been described, but the underlying mechanism remains unclear. The continued presence of MeV RNA in multiple tissues may contribute to immune suppression. Many viruses that cause immune suppression can infect cells of the immune system [154]. MeV can infect T cells, B cells, and monocytes and we have observed the prolonged presence of MeV in each of these immune cells.

MeV RNA persistence may play an important role in sustaining and driving antibody responses, leading to the establishment of life-long immunity. In the case study presented in Chapter 5, we observed that the lack of a robust MeV-specific antibody response correlated with undetectable levels of MeV RNA in multiple tissues. We have also shown that sustained levels of MeV RNA in lymphoid tissue are coincident with ongoing proliferation and Tfh cell expansion.

Viral RNA persistence has been described for animal models of acute viral encephalitis [197], hepatitis, and arthritis [198]. Rhinovirus and enterovirus RNA persistence has also been described in children for up to five weeks after the acute phase of infection [199]. While the consequences of viral RNA persistence for some infections have implications in pathogenesis, the significance of persistence for many infections, including measles, is still not clear. Our work has begun to provide some insight into the importance of the prolonged presence of MeV RNA.

The mechanism by which MeV RNA is able to persist is still unclear. It is possible that persistence may occur via low-grade replication. The nature of the RNA detected at later time points is still undetermined as our current assay is targeted towards detection of the MeV nucleocapsid (N) gene and does not distinguish between plus- and minus-strand RNA. Therefore, it is unknown whether we are detecting full length RNA or the ratio of genomic (-) versus messenger (+) RNA that is being recovered. Studies in mice revealed that coxsackievirus plus- and minus- strand viral RNAs were present at near equivalent amounts and persisted via the production of a stable double-stranded conformation [200]. In future studies, it will be important to develop a qRT-PCR assay with primers and probes specific for other measles virus genes and strand polarity. If we

detect viral mRNA after the clearance of infectious virus, this would indicate active transcription, and continued translation of protein to stimulate immune cells, occurring during the recovery phase. In addition, this will begin to provide some insight into the molecular mechanism of measles RNA persistence.

The ability to avoid recognition by the immune system is likely to be another critical factor in viral persistence. We observed that MeV RNA preferentially persisted in specific subsets of immune cells, specifically B cells and monocytes. MeV RNA could also be detected in T cells in lymphoid tissue although at much lower quantities. This suggests that viral RNA may be able to persist by "hiding" out in specific immune cell types. Viruses such as cytomegalovirus (CMV) and HIV have been shown to persist in monocytes and macrophages [201]. It will also be interesting to determine whether viral RNA is being detected in specific memory cell populations. Thus, further phenotypic characterization of lymphocyte populations that harbor MeV RNA for extended periods of time is needed to give us better insight into the role and mechanism of RNA persistence.

Innate Immune Response to Measles

The types of innate immune responses induced during measles have not been completely defined. We have shown that *in vivo* infection with wild-type MeV inhibits the induction of type I IFN. This phenomenon is not affected by virus attenuation as type I IFN induction was not observed in monkeys infected with vaccine MeV [35]. The MeV C and V proteins are primarily responsible for the regulation of type I IFNs during measles infection and mutations in the V protein inhibit its ability to block IFN

production [202]. The V proteins of most paramyxoviruses can inhibit IFN induction by directly targeting MDA-5. This supports the observation that the antagonistic function of type I IFN by the V protein requires interaction of the conserved, C-terminus with MDA-5 [203].

Another mechanism that MeV may use to circumvent the IFN response is by limiting the production of PAMPs. The V protein of MeV could function to control virus transcription and regulation by inhibiting the activity of the phosphoprotein (P) or the large protein (L) as described for other paramyxoviruses [36]. The MeV C protein also negatively regulates viral RNA synthesis by inhibiting the RNA polymerase [8]. While it is clear that MeV has evolved strategies to inhibit type I IFN induction, the viral PAMP(s) involved in activating the innate immune response is still unknown. The tightly controlled regulation of type I IFN during measles virus infection suggests that other innate immune responses are important for controlling replication and activation of the adaptive immune response.

Adaptive Immune Response to Wild-type Measles Virus

Many aspects of the development of adaptive immune responses shortly after MeV infection have been well characterized. Recovery from infection with wild-type MeV results in prolonged protective immunity, however the mechanism involved in the induction of this robust immune response is not fully understood. The development of an animal model for measles, specifically the macaque model, has allowed for a more longitudinal analysis of the dynamics of the adaptive immune response during infection. Previous studies in rhesus macaques have only analyzed the development of MeV-

specific immune responses through approximately 3 months post-infection [23]. In our current study, we have described both cellular and humoral responses to measles infection through six-months post-infection. Understanding how long-lived protective immunity is established can have major implications for understanding other viral diseases and vaccine development.

Antibodies to measles virus are important for protection, and virus-specific neutralizing antibody levels correlate with protection from disease [64]. B cells undergo somatic hypermutation in germinal centers, which allows for the selection of cells producing high affinity antibodies for a specific foreign antigen. Our data showed a progressive increase in antibody avidity along with an increase in cellular proliferation in lymphoid tissue, which suggests prolonged germinal center Tfh cell activity and B cell selection through six months post-infection. It will be important in future studies to characterize the evolution of B cell responses in the periphery and Tfh cell responses in lymph node to better understand the development of MeV-specific humoral immunity. We also observed that deficits in antibody responses did not affect virus clearance or disease pathogenesis. This supports an important role for cellular responses in viral immunity, and suggests that both antibody and T cell responses should be studied when assessing vaccine efficacy.

Our study has shown that measles infection induces prolonged multi-phasic virus-specific T cell responses. During the first two weeks following infection T cells are predominantly producing IFNγ followed by a shift to IL-17 production around two to three months post-infection. This shift to IL-17 production during measles convalescence is coincident with the production of Th2-associated cytokines, previously described [80],

and implies a functional change in the antigen presenting cells stimulating differentiation of naïve T cells. These changes in the cytokine profile may, in part, account for the susceptibility of individuals to secondary infections following MV infection, and may also promote B cell maturation and prolonged production of antibody secreting cells. We also observed late production (~5-6 months p.i.) of IFNγ and IL-17 by T cells which may represent memory populations, especially because there is growing evidence of the existence of Th17 memory cells in addition to effector and central memory populations [194]. Further phenotypic analysis of T cell populations by FACS analysis would be needed to gain a more detailed explanation of the cellular sources of late IFNγ and IL-17 production.

The exact role of Th17 responses during MeV infection is still unknown and this is one of the first studies to explore the dynamics of Th17 responses through six-months post-infection. Tregs prevent excessive effector T cell activation [204] and can modulate the proinflammatory nature of Th17 cells. In the current study, we did not study the dynamics of Tregs, which also may play an important role in recovery from measles virus infection. In mice, IL-17 can both increase Tregs [175, 205] and inhibit T cell cytotoxicity. In RSV, an imbalanced Th17/Treg response can result in exacerbation of lung inflammation and more severe disease [204]. During the response to MeV, Foxp3 expression in PBMCs is low 7-14 days after infection, then increases and is sustained once infectious virus is cleared [23]. Therefore, it is possible that the balance between generation of Tregs and Th17 cells after the acute phase of infection promotes recovery and affects the process of MeV RNA clearance.

Adaptive Immune Response to Measles Vaccine

The most effective means of preventing measles is through immunization with a live-attenuated virus. Measles vaccines induce both humoral and cellular immune responses, and the duration of vaccine-induced immunity is at least several decades if not longer. While some correlates of protection from measles have been identified, the mechanism behind vaccine-induced protective immunity is still poorly understood. Furthermore, the durability of protection has been questioned due to reports of measles outbreaks in vaccinated populations [206, 207].

We participated in a study assessing the adaptive immune response in adolescents five years after receipt of a third dose of MMR. We observed MeV-specific IFNγ-producing T cell responses that were predominantly specific for the H protein. Previous studies have shown that vaccination with alphavirus replicon particles expressing H (VEE/SIN-H) induced protective immunity [208]. This vaccine induced a Th1 cytokine bias following challenge, in contrast to the Th2 cytokine bias induced by a non-protective vaccine [208]. Thus, H-specific Th1 responses are likely an important contributor in MeV-induced vaccine immunity. Collectively these data offer new approaches to vaccine strategies, suggest T cell responses are important for vaccine immunity, and provide additional parameters for assessing vaccine efficacy.

Previous reports from the cohort included in our study observed no increase in T-cell responses at neither 1-month nor 1-year post-MMR3 receipt compared to baseline [191]. These data suggest that a third dose of vaccine may not effectively boost measles immunity. However, various studies have reported a waning in measles-specific antibody

titers and T cell responses [189, 209]. Therefore, alternative strategies should be considered to solve the problem of waning immunity.

The overall goal of our study was to characterize the dynamics of MeV clearance and several aspects of the immune response to wild-type measles. We hypothesized that a primary wild-type measles infection would be characterized by a failure of the innate immune response to induce IFN, but would result in viral RNA persistence predominantly in B cells from PBMCs of blood and lymphoid tissue. In general, we expected the MeV-specific humoral response to be rapid, stable and prolonged, while, the virus-specific cellular immune response would be more dynamic exhibiting peak functionality at earlier time points (2-8 weeks) post-infection.

We have demonstrated that the innate immune response to measles does not involve the induction of type I interferon. However, primary infection results in the prolonged presence of viral RNA in multiple tissues and various immune cell types. Viral RNA persistence was especially prolonged in lymphoid tissue and predominantly in antigen presenting cells. In correlation with this, we saw prolonged measles-specific humoral and cellular immune responses through six-months post-infection.

We speculate that viral RNA may be able to persist by existing in a more stable or higher order conformation. As a result, the prolonged presence of viral RNA, and possibly protein, in lymphoid tissue after WT MeV infection leads to continuous stimulation of antibody and T cell responses that result in life-long immunity. In addition, we observed the ongoing evolution of the virus specific cellular immune responses during MeV RNA clearance, which suggests that multifunctional T cells may regulate immune-

mediated clearance. A better understanding of the evolution of ASC and GC responses in persistently infected lymph nodes, will help to inform the induction of long-lived plasma cells and persistent high levels on antibody for development of other vaccines.

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Aug 2004- May 2008	**B.S. in Forensic Science** (Concentration: Forensic Biology) Virginia Commonwealth University, Richmond, VA

Additional Research Experience

June 2007- Aug 2007	**National Science Foundation Research Experiences for Undergraduates,** San Jose State University, San Jose, CA

Honors, Grants, and Awards

2013-2014	Ruth G. Wittler Student Scholarship
2012-2013	Dr. Bettylee Hampil Fellowship
2011	Towson University Graduate Student Association Award
2009-2011	Towson University Graduate Assistantship
2010	Beta Beta Beta National Biological Honor Society
2007	SACNAS National Conference Travel Grant

Professional Societies

2012- present	American Society for Microbiology
2011- present	American Society of Virology
2014	Association for Women in Science

Teaching Experience

Fall 2016	**Teaching Assistant,** Johns Hopkins University Bloomberg School of Public Health Course Title: Public Health Perspectives on Research
Spring 2016	**Teaching Assistant,** Johns Hopkins University School of Nursing Course Title: Introduction to Nutrition
Fall 2015	**Teaching Assistant,** Johns Hopkins University School of Nursing Course Title: Introduction to Nutrition
Fall 2014	**Teaching Assistant,** Johns Hopkins University Bloomberg School of Public Health Course Title: Public Health Perspectives on Research
Fall 2009-2012	**Teaching Assistant**- Towson University Course Title: Introductory Biology Lab for non-science majors
Fall 2005- Spring 2006	**Teaching Assistant**- Virginia Commonwealth University, Ram Pal Program Course Title: Introduction to the University

Abstracts

Nelson, A.N., Putnam N., Jamil A., Baxter V., Adams R.R., and D.E. Griffin. Viral RNA Persistence during a Primary Wild-type Measles Infection. Within host RNA virus persistence: mechanisms and consequences meeting. St Andrews, Scotland (2016). Oral presentation.

Nelson, A.N., Putnam N., Jamil A., Baxter V., Adams R.R., and D.E. Griffin. Viral RNA Persistence and the Evolution of Measles-specific Humoral Immunity in Rhesus Macaques. American Society of Virology. Blacksburg, VA (2016). Oral presentation.

Nelson, A.N., Putnam N., Baxter V., Adams R.J., and D.E. Griffin. Measles Virus-Specific T Cell Responses and their Association with Viral Control and Clearance. 4th Measles Rubella Symposium. Decatur, GA (2015). Oral presentation.

Nelson, A.N., Putnam N., Baxter V., and D.E. Griffin. Functionality of Measles Virus-Specific T Cell Responses and their Association with Viral Control and Clearance. Delta Omega Poster Competition. Baltimore, MD (2015). Poster presentation.

Nelson, A.N., Putnam N., Baxter V., and D.E. Griffin. Functionality of Measles Virus-Specific T Cell Responses and their Association with Viral Control and Clearance. American Society of Virology. Western University, London, Ontario, Canada (2015). Poster presentation.

Nelson, A.N., Lijewski, S.A., and B.J. Margulies. Development of a biodegradable subcutaneous implant containing acyclovir for the long-term suppression of HSV-1 reoccurrences. American Society of Virology. Madison, WI (2012). Poster presentation.

Nelson, A.N., S.A. Lijewski, and B.J. Margulies. Development of a Biodegradable Subcutaneous Implant Containing Acyclovir for the Long-Term Suppression of HSV-1 Recurrences. 8th Annual Regional Microbiology Educators Network Student Research Symposium, Swarthmore, PA (2011). Poster presentation.

Nelson, A.N., Lijewski, S.A., and B.J. Margulies. Development of a biodegradable subcutaneous implant containing acyclovir for the long-term suppression of HSV-1 reoccurrences. 2011 Annual Biomedical Research Conference for Minority Students. St. Louis, MO (2011). Poster presentation.

Nelson, A.N. and J. Soto. Determining if the AREI sites in the exon-coding sequence of *Hro-Twist* play a role in mRNA stabilization in *Helobdella robusta* embryo. 2007 SACNAS National Conference. Kansas City, MO (2007). Poster presentation.

Publications

Griffin DE, Lin W, and **AN Nelson.** 2017. The Causes and Consequences of Measles Virus Persistence. F1000 Research (In press).

Nelson AN, Putnam N, Hauer D, Baxter VK, Adams RJ and DE Griffin. The Prolonged Evolution of T cell Responses during Measles Virus Infection and RNA Clearance. (Submitted to Scientific Reports).

Wen-Hsuan W. Lin, **Nelson AN**, Ryon J.J, Moss WJ, and DE Griffin. 2017. Plasma cytokines and chemokines in Zambian children with measles: innate responses and association with HIV-1 co-infection and in-hospital mortality. J Infect Dis.

Kulcsar KA, Baxter VK, Abraham R, **Nelson AN**, Griffin DE. 2015. Distinct immune responses in resistant and susceptible strains of mice during neurovirulent alphavirus encephalomyelitis. J Virol 89:8280–8291. doi:10.1128/JVI.00173-15.

References

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